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Biosensor assay for determination of haptoglobin in bovine milk

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Despite more than 30 years of research into mastitis diagnostics, there are few alternatives to the somatic cell count (SCC) in practical use for identification of cows with subclinical mastitis. Mastitis is not only an animal welfare problem, but also affects the yield, composition and technological properties of milk. Hence, dairy cooperatives give farmers a premium quality payment to encourage low SCC although there is no clear scientific data defining the level of SCC in bulk tank milk that is associated with additional benefits in terms of milk quality. Recent research on alternative markers for inflammatory reactions in the lactating cow, e.g. in mastitis, includes investigations of the acute phase protein, haptoglobin (Hp). So far, the content of Hp in milk has mainly been studied in relation to mastitis diagnostics, with little attention given to its importance for milk composition and technological properties. At present, Hp in milk is measured using ELISA, but this technique is not suitable for routine large-scale analysis. In recent years, optical biosensor technology has been used for automated and rapid quantitative analysis of different components in milk, but so far not for analysis of acute phase proteins. The aim of the present study was to develop a rapid and sensitive biosensor method to determine Hp in milk. An affinity sensor assay based on the interaction between Hp and haemoglobin was developed using surface plasmon resonance (SPR) biosensor technology. The assay was used to analyse Hp in composite milk samples from cows without any clinical signs of mastitis and quarter milk samples with a weak to strong reaction in the California Mastitis Test (CMT). A commercial ELISA for determination of Hp in milk was used for comparison. The limit of detection (LOD) of the biosensor assay was determined as 1·1 mg/l. Within-assay and between-day variations were determined both with bulk tank milk spiked with human Hp and with composite milk samples containing bovine Hp. Coefficients of variation varied between 3·6 and 8·6% at concentrations between 4·0 and 12 mg/l, respectively. Agreement between the results obtained by the biosensor assay and the ELISA was satisfactory; however, the results obtained by the biosensor were generally lower than the results obtained by the ELISA. Possible explanations for this observation are discussed.

Keywords: Mastitis diagnostics, dairy cow.

In spite of more than 30 years of research into mastitis diagnostics, the milk somatic cell count (SCC) is still the standard method used to identify cows and udder quarters with subclinical mastitis. Besides being an animal welfare problem, mastitis affects the yield, composition and processing properties of the milk, with a negative impact on the quality of the final dairy product (Roux et al. 2003). SCC is therefore also used to assess milk quality at the bulk tank level and according to EC Directive 853/2004, the SCC of the bulk tank milk must be <400 000 cells/ml (EC, 2004). Despite a lack of unambiguous scientific evidence showing at what level of SCC the bulk tank milk composition is negatively affected, the pressure on milk producers to lower the SCC below present standards has increased. In Sweden, dairy co-operatives generally give a bonus payment when the SCC is <150 000–200 000 cells/ml. With increasing herd size and number of automated milking systems there is a demand for rapid, large scale analysis of milk to allow early identification of infected quarters (Grönlund et al. 2003; Pyörälä, 2003). Various techniques have been suggested and evaluated as alternatives to SCC, including changes in serum proteins, enzymes, ions and lactose (Pyörälä, 2003). At present,

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electrical conductivity is the only method used commercially in automated milking systems despite the fact that electrical conductivity is not considered to be sensitive enough to detect subclinical mastitis (Nielen et al. 1995).

In the last decade there has been an increasing interest in the possibility of using acute phase proteins (APP) in veterinary diagnosis. APP are a group of blood proteins that change dramatically in concentration upon challenge, e.g. infection, inflammation or trauma. The proteins can be classified according to the magnitude of their increase (positive APP) or decrease (negative APP), and the response pattern of APP is species specific. One of the major APP in cattle is haptoglobin (Hp). Hp is mainly produced by hepatocytes and its synthesis increases upon stimulation by different cytokines (Gruys et al. 1994; Murata et al. 2004; Petersen et al. 2004). Measured in serum, Hp is an unspecific marker of disease as an increased concentration can be a result of a number of causes. In mastitis, the permeability between the milk and blood compartments increases and hepatically derived Hp can enter the milk (Eckersall et al. 2001). Hp in milk thus becomes a specific marker of mastitis, since the Hp concentration in milk from healthy quarters is low or undetectable (Eckersall et al. 2001; Grönlund et al. 2003; Grönlund et al. 2005). Recent research indicates that Hp may also be synthesized locally in the mammary gland, as Hiss et al. (2004) demonstrated Hp mRNA expression in mammary gland tissue by real-time PCR.

Hp in milk is commonly determined by commercially available or in-house immunoassays. Many of these assays become cumbersome and time consuming when used for large-scale analysis. Compared with human medicine, there is a lack of APP tests for routine analysis in veterinary medicine (Eckersall, 2004). During the last decade, several applications of optical biosensor technology for quantitative analysis of different components and residues in milk have been described (Baxter et al. 2001; Gillis et al. 2002; Indyk et al. 2002; Samsonova et al. 2002; Nygren et al. 2003; Dupont et al. 2004; Gustavsson et al. 2004; Haasnoot et al. 2004). The main advantage of the biosensor over ELISA, is the automated and rapid analysis, each sample requiring approximately 5–10 min. The aim of the present study was to develop an optical biosensor assay for Hp based on the strong interaction between haemoglobin and Hp. The resulting assay will in our future work be used to determine whether APP, in addition to being used as indicators of udder health, also may function as biomarkers for raw milk quality.

Materials and Methods

Instrumentation

The optical biosensor used was a Biacore Q instrument (Biacore AB, SE-754 50, Uppsala, Sweden) with Biacore Q Control Software (version 3.0.1) for instrument operation and BIA evaluation (version 3.2) for data handling.

Reagents

Sensor Chip CM5 (research grade), HBS-P buffer pH 7·4 (10 mM 4-[2-hydroxyethyl] piperazine-1-ethane-sulphonic acid (Hepes), 150 mM-NaCl, 0·005% (v/v) surfactant P-20), amine coupling kit (400 mM-N-ethyl-N′-(3-ethylaminopropyl) carbodiimide hydrochloride (EDC), 100 mM-N-hydroxysuccinimide (NHS) and 1 M-ethanolamine hydrochloride) and SDS (0·5% (w/v) sodium dodecyl sulphate) were obtained from Biacore AB (SE-754 50, Uppsala, Sweden).

Owing to the unavailability of bovine Hp at the time this study was initiated, the human form of the protein was used (Biogenesis, Poole, UK). Bovine haemoglobin was obtained from Sigma (St Louis MO, USA). Before use, haemoglobin was stabilized in a buffer containing $K_3Fe(CN)_6$ (200 mg/l), KCN (50 mg/l) and KH_2PO_4 (140 mg/l). Acetate buffer (0·01 M, pH 3·8) was used for immobilization of Hp to the sensor surface and PBS (0·01 M, pH 7·4) was used for dilution of Hp during preparation of the Hp standards.

Milk samples

Bulk tank milk and cow composite milk samples (*n*=43) from clinically healthy cows, i.e. cows with no observable signs of any disease, were collected from the University dairy herd. Quarter milk samples (*n*=28) with weak to strong reactions in the California Mastitis Test (CMT) were provided by the National Veterinary Institute (Uppsala, Sweden). Most of the latter samples (25 out of 28) were bacteriologically positive. Since the biosensor method is based on the interaction between added haemoglobin and Hp, haemoglobin from blood will disturb the analysis. Consequently, no milk samples that by visual assessment were concluded to contain blood were included in the study. Milk samples were defatted by centrifugation for 5 min at 750 ***g*** and stored at −70 °C until analysed.

Haptoglobin ELISA

A commercial Hp-ELISA (Phase™ Bovine Haptoglobin Enzyme Immunoassay, Tridelta Development Ltd, Bray, Co. Wicklow, Ireland) was used for comparison with the biosensor assay. In the ELISA, the wells are coated with haemoglobin that will bind free Hp in the sample. A Horse Radish Peroxidase (HRP)-labelled anti-Hp monoclonal antibody is added, which will bind to the captured Hp. Finally, a tetramethyl benzidine (TMB) substrate solution is added, and the colour intensity obtained is proportional to the concentration of Hp in the sample. The test was performed according to the manufacturer's instructions and the claimed limit of detection (LOD) of the ELISA was 0·3 mg/l.

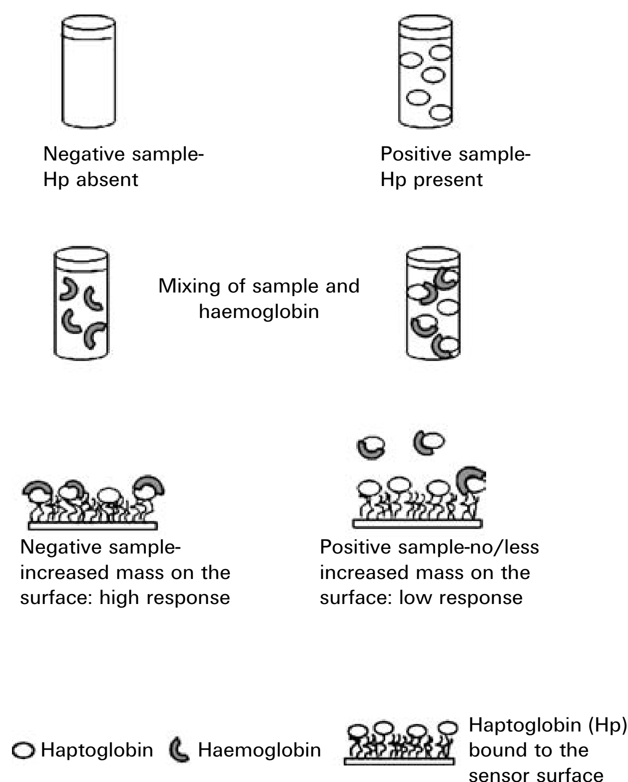

Fig. 1. Schematic illustration of the competitive biosensor assay principle. The milk sample is mixed with haemoglobin before injection over a sensor surface with haptoglobin (Hp) immobilized. With no Hp present in the sample, the haemoglobin will bind to immobilized Hp on the sensor surface. When Hp is present in the sample, it will form a complex with added haemoglobin and inhibit haemoglogin-binding to the surface. The amount of haemoglobin binding to the surface is therefore inversely proportional to the amount of Hp in the sample.

Haptoglobin biosensor assay

Principle of assay. This competitive assay is based on the strong interaction between Hp and haemoglobin, and the principle is explained in Fig. 1. The milk sample is mixed with bovine haemoglobin before injection over a sensor surface with immobilized Hp. When no Hp is present in the sample haemoglobin will bind to immobilized Hp on the sensor surface. Since the detection principle is based on mass-dependent changes in refractive index at the sensor surface, haemoglobin binding to the surface results in an increased response. When Hp is present in the sample, it will form a complex with added haemoglobin and inhibit haemoglobin binding to the surface. The amount of haemoglobin binding to the surface is therefore inversely proportional to the amount of Hp in the sample.

Preparation of biosensor surface. Haptoglobin (500 mg/l) in 0·01 M-acetate buffer pH 3·8 was immobilized to a CM5 sensor chip using the standard amine coupling procedure with a constant flow rate of 5 μl/min during the entire immobilization procedure. Briefly, the carboxymethylated dextran surface was activated by injection of a mixture containing equal amounts of EDC (400 mM) and NHS (100 mM) for 7 min. The ligand, i.e. Hp, was injected for 7 min and the NHS-esters formed during activation reacted with primary amino groups of the ligand. After coupling, remaining active NHS-esters were deactivated with 7 min injection of 1 M-ethanolamine.

Preparation of standards. Hp was dissolved in 0·01 M-PBS pH 7·4 and added to bulk tank milk in different concentrations. The stock solution (1000 mg/l) was used to prepare milk samples with final Hp concentrations of 1·25, 2·5, 5, 10, 20 and 40 mg/l for construction of a standard curve.

Analysis of composite and quarter milk samples. Frozen milk samples were thawed at room temperature before analysis. Milk samples were pipetted into a microtitre plate and automatically mixed with haemoglobin diluted in HBS-P buffer (2·5 mg/l). The mixture (10% sample and 90% haemoglobin solution) was then injected (30 μl) over the sensor surface. After each measurement the surface was regenerated by injection of 30 μl of 2·1 mM-SDS. HBS-P was used as running buffer at a flow rate of 30 μl/min throughout the analysis.

To see whether variations in the sample matrix would affect the results, all milk samples were initially diluted with bulk tank milk negative in the Hp-ELISA before analysis (1 : 2, 1 : 5, 1 : 10, 1 : 20 etc. depending on Hp concentration in the sample). For samples with slightly elevated concentrations (1–5 mg/l) it was found that this procedure was not necessary, since there was no observable matrix effect, and the result after the first dilution step was the same as the result for the undiluted sample. For samples with very high Hp levels, usually samples from cows with clinical mastitis, this procedure was necessary both to fall within the linear part of the standard curve and to overcome effects from the matrix.

Evaluation of assay performance. Hp standards were analysed according to the assay procedure and the results were used to construct calibration curves. Assay LOD was determined by analysing 20 replicates of a bulk tank milk sample negative in the Hp ELISA (<0·3 mg/l). LOD was defined as the lowest concentration corresponding to a relative response significantly different (3 × SD) from the average response of 20 Hp-negative samples. The precision of the assay was determined by analysis of bulk tank milk spiked with human Hp at two different concentrations (4·0 and 12 mg/l) and two composite milk samples containing bovine Hp (4·1 and 7·2 mg/l according to ELISA). Ten replicates of each milk sample were analysed once daily for three consecutive days. The SD

Table 1. Precision of the haptoglobin (Hp) biosensor assay determined within assay and between days by analysis of bulk tank milk spiked with human Hp at two different levels (4 and 12 mg/l) and two cow composite milk samples containing 4·1 and 7·2 mg/l of bovine Hp according to ELISA

	Bulk tank milk spiked with human Hp		Cow composite milk sample	
	4·0 mg/l	12·0 mg/l	4·1 mg/l	7·2 mg/l
Within assay (*n*=*10*)				
Mean value (mg/l)	4·8	12·6	4·1	6·4
SD (CV%)	0·17 (3·6)	0·70 (5·5)	0·18 (4·3)	0·44 (6·8)
Between days (*n*=*3*)				
Mean value (mg/l)	4·5	11·8	4·1	6·5
SD (CV %)	0·26 (5·9)	1·01 (8·6)	0·24 (5·9)	0·29 (4·5)

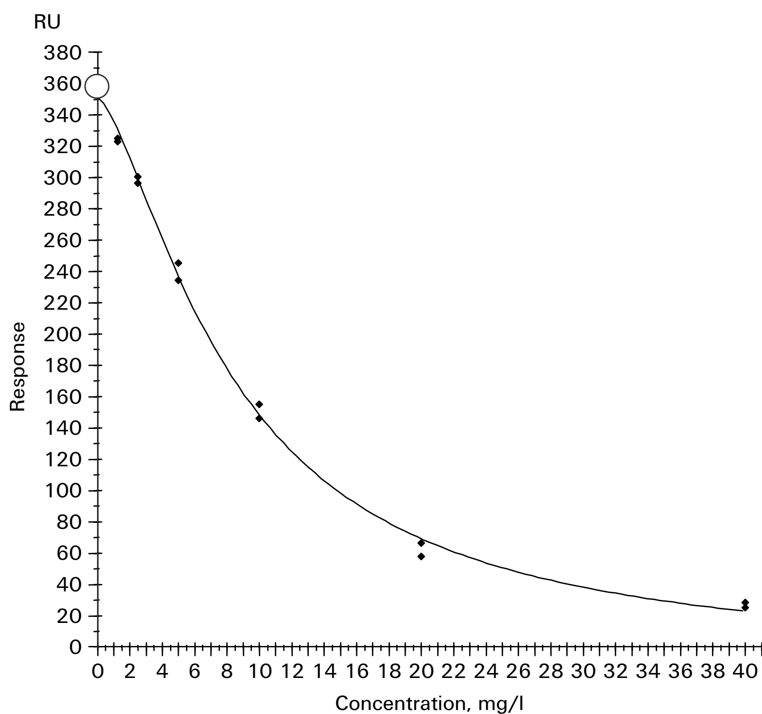

Fig. 2. Haptoglobin (Hp) calibration curve obtained by duplicate analysis of bulk tank milk spiked with human Hp using the biosensor assay.

for the analysed concentrations was determined within and between days.

The agreement between results obtained with the biosensor assay and the ELISA was evaluated using Wilcoxon single rank test (Minitab Inc., version 14, 2003, State College, PA, USA).

Results

A typical calibration curve for the Hp biosensor assay is presented in Fig. 2. The average response in the analysis of Hp-negative samples ($n=20$) was 403±3 RU, resulting in a background level of 0·2±0·3 mg/l Hp and a detection limit of 1·1 mg/l. The CV of the assay varied between 3·6% and 6·8% within assay, and between 4·5% and 8·6% between days (Table 1) for bulk tank milk spiked with human Hp (4 and 12 mg/l) and cow composite milk samples with bovine Hp (4·1 and 7·2 mg/l).

Of the analysed composite milk samples from cows with no observable signs of mastitis, 36 out of 43 samples were Hp-negative in both ELISA and biosensor method. In the remaining 7 samples, the ELISA detected concentrations varying between 1 and 12 mg/l, whereas the biosensor assay only detected Hp in the 3 samples with the highest concentrations as measured by the ELISA (Table 2).

When analysing quarter milk samples with weak to strong CMT reactions, 24 out of 28 samples had Hp concentrations <50 mg/l whereas the remaining 4 samples contained higher Hp concentrations (Table 3). Results

Table 2. Haptoglobin (Hp) determined with the biosensor assay and the ELISA in composite milk samples from cows with no clinical signs of mastitis

	Haptoglobin (mg/l)	
No. of samples	ELISA	Biosensor
36	<LOD	<LOD
1	12	7
1	7	4
1	3	2
1	2	<LOD
3	1	<LOD

Table 3. Haptoglobin (Hp) determined with the biosensor assay and ELISA in quarter milk samples from cows with weak to strong reactions (1–3) in the California Mastitis Test (CMT)

	Haptoglobin (mg/l)		
Milk sample ID	ELISA	Biosensor	CMT score
2060.4	154	143	2
2045.2	120	77	3
2060.3	106	93	3
2045.3	86	51	2
2067.2	46	24	2
2024.2	43	20	3
2048.2	40	27	1
2058.3	38	26	3
2038.3	36	25	3
2020.3	25	23	3
2043.1	20	14	2
2023.3	15	9	2
2039.2	12	4	2
2016.3	10	5	2
2036.2	10	18	3
2045.4	10	8	1
2039.4	9	6	3
1602.2	8	8	2
2016.4	7	8	2
2070.3	4	3	2
2037.3	3	2	2
2044.1	3	2	3
2068.2	3	1	2
1603.2	2	2	2
2022.3	2	1	3
1564.4	1	2	1
1619.2	0·5	0	3
1600.3	0·5	0	3

obtained by the biosensor assay were generally lower than by ELISA, and the difference between the two methods seemed to increase with increasing Hp concentrations (Fig. 3).

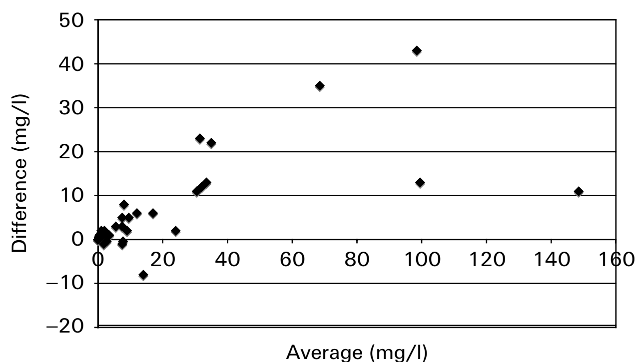

Fig. 3. Bland-Altman plot illustrating the agreement between the ELISA and biosensor assays in the determination of haptoglobin (Hp) in milk. Average Hp concentration and the difference between ELISA and biosensor assay on x-axis and y-axis, respectively.

Discussion

To our knowledge, this is the first application of optical biosensor technology for determination of APP. The first commercially available test kit for Hp analysis (Phase™ Range Haptoglobin Assay, Tridelta Development Ltd, Bray, Co. Wicklow, Ireland) was introduced in the late 1990s. This method is based on the conserved peroxidase activity of the Hp-haemoglobin complex at low pH. However, this principle makes the method unsuitable for analysis of Hp in milk owing to interference by endogenous lactoperoxidase activity. Recently published investigations of Hp in milk have instead used ELISA based on monoclonal antibodies against bovine Hp (Grönlund et al. 2003; Hiss et al. 2004). The biosensor assay described in this paper is, like the early methods for determination of Hp in serum, based on the strong interaction between Hp and haemoglobin (Makimura & Suzuki, 1982). With the biosensor assay this principle is applicable also to milk, since detection is not based on peroxidase activity but changes in mass on the sensor surface. During development of the biosensor assay both a direct assay with haemoglobin on the surface, and a competitive assay, adding known amounts of haemoglobin to the sample before injection across a Hp surface, were investigated. For unknown reasons, we did not succeed in developing a direct assay. One possible explanation could be that immobilized haemoglobin is poorly exposed on the surface and as a consequence, Hp, which is a much larger molecule, cannot interact with the binding site. This approach would need further work, for example a linker to attach Hp to the surface. In contrast, the development of the competitive assay was quite straightforward and this format was chosen for further studies.

Regeneration of the biosensor surface, i.e. dissociation of the interaction between ligand and the interacting compound, is normally achieved by injection of a buffer with low or high pH. This procedure was not sufficient to dissociate the strong interaction between Hp and haemoglobin and various regeneration solutions were evaluated with little success. Increasing the ionic strength, adding chaotropic agents (NaSCN, guanidine-HCl) and/or

ethylene glycol had no effect or reduced the activity of the surface. Finally, it was found that a weak solution (2·1 mM) of SDS did dissociate the complex efficiently without damaging the immobilized Hp, and this procedure was subsequently used for successful regeneration of the sensor surface.

The ability of the biosensor assay to detect Hp in milk agreed satisfactorily with the ELISA. The precision of the method was high both within assay and between days, but the biosensor assay had a higher LOD (1·1 mg/l) than the ELISA (0·3 mg/l as claimed by the manufacturer). Furthermore, the concentrations obtained by the biosensor were generally lower than the concentrations obtained by ELISA. A Bland-Altman plot of the differences between the two assays (ELISA – biosensor) against the average concentration suggests that the difference between the two tests, as well as the variation, increases with increasing Hp concentration (Fig. 3). Moreover the Wilcoxon single rank test (Minitab Inc., version 14, 2003) points out that the two methods disagree significantly ($P<0{\cdot}001$). One possible explanation might be differences in affinity between bovine haemoglobin and the human form of Hp used in standards and the bovine form of Hp present in milk. The human Hp consisted of a mixture of the three human phenotypes, Hp 1-1, Hp 2-1 and Hp 2-2. Bovine Hp, which is highly polymerized with a molecular mass exceeding 1000 kDa (Eckersall & Conner, 1990; Morimatsu et al. 1991), is considered to be most similar to human Hp 2-2 (Eckersall & Conner, 1990). In on-going studies we have therefore used the human Hp 2-2 both as ligand and in standards to see whether the differences between ELISA and biosensor assay will decrease. Moreover, after this manuscript was submitted, bovine Hp also has become commercially available. Our preliminary results with different Hp forms, however, do not suggest that the difference between ELISA and biosensor assay would depend on which Hp form is used as calibrator.

Another, perhaps more likely reason for the difference between the two methods, is that even the smallest blood impurities in the milk may interfere with the biosensor assay by reducing the inhibitory effect of free Hp on the fixed amount of added haemoglobin. Therefore samples containing blood cannot be analysed with this method and for this reason such samples always have to be eliminated. Although the samples in this study were visually examined for the presence of blood, it cannot be excluded that even the slightest elevation of haemoglobin will affect the assay. The fact that the difference in results between two methods increases at high levels (Fig. 3) could indicate that samples associated with clinical cases of mastitis are more likely to be contaminated with haemoglobin. Consequently, underestimation is more likely to occur with samples containing high Hp levels than with samples containing low concentrations. It is clear that the developed assay is not optimal for quantitative determination of Hp, but it can definitely be used as a tool for semi-quantitative determinations, especially with milk from cows with no observable signs of mastitis.

Application of this type of assay is dependent on the outcome of studies on what these proteins, say in relation to milk composition. This is our first manuscript resulting from such studies and it is, therefore, too early to say anything about the usefulness of the assay. If our future studies were to show a strong correlation between elevated levels of Hp and changes in milk composition, then the assay could be used for raw milk quality assessment in the laboratory or on farm. In the last decade, research related to APP in food-producing animals has focused on the diagnostic value of the proteins. We consider our work, focusing on food quality and food safety, to be complementary to these studies.

In conclusion, we describe a simple and rapid method to determine Hp in bovine milk. It is the first biosensor assay to measure Hp and it is based on the strong interaction between Hp and haemoglobin. The biosensor assay has potential to be useful in screening of Hp in milk samples if future studies show that elevated levels of Hp correlate with altered milk composition and impaired milk quality.

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