clear that there is a large variation in the progesterone profiles of different cows and that a fixed threshold on the progesterone level would not provide good heat detection results. However, the investigated approach which uses model parameters rather than a threshold allows to accurately describe the individual profiles and provide personalized detection of heat, pregnancy and abnormalities.

In the future, this approach for data management at the cow level will be applied to the data acquired with the FO-SPR progesterone sensor and the Vis/NIR milk composition sensor to develop a valuable decision support system for dairy farmers.

Keywords. Progesterone, nutritional status, management support.

DEVELOPMENT OF A NEW IMMUNORADIOMETRIC ASSAY FOR PREGNANCY-ASSOCIATED GLYCOPROTEINS (IRMA-PAG) ALLOWING PREGNANCY FOLLOW-UP IN CATTLE BY USING MILK SAMPLES

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Pregnancy-Associated Glycoproteins (PAGs) were first described as placental antigens that were also present in the peripheral circulation of the mother soon after implantation. They are synthesized by the mono- and binucleate thophoblastic cells, some of them being secreted in maternal blood from the moment when the conceptus becomes more closely attached to the uterine wall and formation of placentomes begins. In cattle, concentrations of PAG are detectable in maternal blood from day 28 to day 30 after fertilization. Milk concentrations are 20-30 times lower than in blood samples and cannot be measured by classical radio-immunoassay systems. In the present study we report the use of a highly sensitive immunoradiometric assay for PAG (PAG-IRMA) allowing pregnancy follow-up in cattle by measuring concentrations in milk samples. In the IRMA system, an antibody was coated to StarNunc tubes and used for the immobilization of the antigen. The sample (volume until 4 ml) was incubated in the tube. After incubation, the whole sample was eliminated by several washes. Thereafter, another antibody bound to biotin (detection antibody) was added in order to quantify the antigen present in the sample. After a second wash, the reaction was revealed by adding a Streptavidin tracer (^{125}I). Purified bovine PAG 67kDa was used as standard at concentrations ranging from 100 to 50,000 pg.ml^{-1}. Highly purified immunoglobulins (hp-Ig) were obtained from two distinct rabbit polyclonal antisera by using a specific affinity chromatography (anti-PAG 4B-Sepharose gel). The hp-Ig708 (purified from polyclonal antiserum raised against caprine PAG 55kDa+59kDa) was used as capture antibody (0.01 µg/tube). The hp-Ig727 (purified from polyclonal antiserum raised against purified boPAG67kDa) was used as detection antibody (1:8,000). Radiolabeled streptavidin (125I-Strep; 50,000 cpm·100 µl^{-1}) was used to reveal the Ab-Ag-Ab-Biot complexes. Milk was collected from pregnant cows (n = 20) during the whole duration of lactation until dry-off. Samples were frozen until assay. Before analysis, milk samples were thawed at 37 °C, centrifuged (2,500 x g) and fat was removed. Samples giving high PAG concentrations were serially diluted in order to fit with standard curve range. In pregnant cows, milk PAG concentrations increased from week 10 (56.9 ± 13.1 pg·ml^{-1}) to week 11 (93.5 ± 20.4 pg·ml^{-1}) and week 12 (135.2 ± 27.7 pg·ml^{-1}). Thereafter, PAG concentrations increased regularly until week 32 (2,177.6 ± 496.2 pg·ml^{-1}) and slightly decreased until dry-off at week 35 (1,615.9 ± 663.9 pg·ml^{-1}). Immediately after parturition, PAG concentrations reached 5,615.3 ± 615.7 pg·ml^{-1} and decreased continuously until week 11 postpartum (36.6 ± 2.1 pg·ml^{-1}). In non-pregnant cows, concentrations remain lower than 40-50 pg·ml^{-1} at all time points. In conclusion, a new IRMA-PAG is available for quantitative measurement of PAG concentrations in cattle. This new test can be used for pregnancy diagnosis and follow-up in cattle.

Keywords. Pregnancy diagnosis, pregnancy-associated glycoprotein, dairy cattle.

VALIDATION OF A NEW IMMUNORADIOMETRIC ASSAY (IRMA) ALLOWING QUANTIFICATION OF PREGNANCY-ASSOCIATED GLYCOPROTEINS CONCENTRATIONS IN BOVINE MILK

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Pregnancy-Associated Glycoproteins (PAGs), also known as pregnancy-specific protein B (PSPB) are used since early eighties as pregnancy markers in cattle and other ruminant species. Until now, they are mainly assayed in plasma or serum samples by using radioimmunoassay or ELISA systems. The aim of this work was to develop a very sensitive and robust immunoassay offering the possibility to quantify PAG in milk samples: the PAG-immunoradiometric assay. In the IRMA system, an antibody was coated to StarNunc tubes and used for the immobilization of the antigen. The sample (volume until 4 ml) was incubated in the tube. After incubation, the whole sample was eliminated by several washes. Thereafter, another antibody bound to biotin (detection antibody) was added in order to quantify the antigen present in the sample. After a second wash, the reaction was revealed by adding a Streptavidin tracer (^{125}I). The validation of a highly sensitive immunoradiometric assay for PAG (PAG-IRMA) allowing PAG quantification in bovine milk is reported in details. Purified bovine PAG 67kDa was used as standard at the concentrations ranging from 100 to 50,000 pg·ml^{-1}. Highly purified immunoglobulin hp-Ig708 (purified from polyclonal antiserum raised against caprine PAG 55kDa+59kDa) was used as capture antibody (0.01 μg/tube). The hp-Ig727 (purified from polyclonal antiserum raised against purified boPAG67kDa) was used as detection antibody (1:8,000). Radiolabeled streptavidin (125I-Strep; 50,000 cpm·100 μl^{-1}) was used to reveal the Ab-Ag-Ab-Biot complexes. Minimum detection limit was calculated as the mean concentration minus twice SD of 10 duplicates of the zero (B0) standard. Reproducibility was determined by calculating the intra- and inter-assay coefficients of variation (CV). The accuracy was determined by adding increasing quantities of purified boPAG67kDa (10, 20, 40 and 80 pg) to bovine milk containing low PAG concentrations. Specificity was tested regarding 16 different compounds including carbohydrates, aspartic proteinases, hormones and major milk proteins. These molecules were tested at dilutions ranging from 0.1 ng·ml^{-1} to 100 μg·ml^{-1}. Parallelism was assessed by serially diluting pregnant cow milk containing relatively high PAG concentrations. Minimum detection limit was 4 pg·ml^{-1}. Intra- and inter-assay CV ranged from 13.7 to 15.8%. Accuracy (recovery of added PAG) ranged from 86.3 to 104.8%. No cross reaction was observed with almost all tested compounds except lactoferrin (2.4%), rennin (2.5%), porcine pepsinogen (2.3%) and porcine pepsin (5.2%) at 100 μg·ml^{-1} (most of them at supra-physiological levels). Dilutions of bovine milk containing detectable PAG concentrations were parallel to standard curve. In conclusion, the new PAG-IRMA is highly sensitive, accurate, precise and reproducible for PAG measurements in bovine milk samples. Parallelism test reveals an excellent ability to quantify PAG concentrations in milk of dairy cattle. This new test can be an alternative method useful for pregnancy diagnosis and follow-up in bovine species. **Keywords.** Pregnancy-associated glycoprotein, immunoradiometric assay, dairy cattle.

UPLC®-MS/MS, AN ANALYTICAL TOOL FOR THE ACCURATE AND RAPID QUANTIFICATION OF PHYTOESTROGEN METABOLITES IN MILK

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The term "phytoestrogen" is used to define a wide variety of nonsteroidal compounds that occur naturally in many plants. When they are absorbed by cows, these polyphenolic compounds undergo biotransformation by specific bacteria present in the gastrointestinal tract. The resulting metabolites are absorbed in the organism and some are excreted in milk. The impact of these compounds on human health divides opinion. Some scientists believe that they might have adverse health effects. Others believe the opposite, especially with regard to their microbial metabolites, such as equol. Whatever one's view on the subject, scientists need accurate, sensitive and rapid analytical methods in order to continue the research on clarifying the phytoestrogen issue. Cow's milk is an animal product that is common in the human diet, and it is therefore important to evaluate its content of phytoestrogen metabolites. In order to study the human intake of equol *via* commercial milk, an analytical method was developed and validated following EMA/CVMP/VICH/463202/2009 guidelines. Enzymatic hydrolysis was used to release the equol. It was then extracted using double liquid/liquid extraction and analyzed

Validation of a new immunoradiometric assay (IRMA) allowing quantification of Pregnancy-Associated Glycoproteins concentrations in bovine milk

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① Introduction

• Pregnancy-Associated Glycoproteins (PAGs), also known as pregnancy-specific protein B (PSPB) are used since early eighties as pregnancy markers in cattle and other ruminant species.

• Until now, PAGs are mainly assayed in plasma or serum samples by using radioimmunoassay or ELISA systems.

② Aim

The aim of this work was to develop a very sensitive and robust immunoassay offering the possibility to quantify PAG in milk samples: the PAG-immunoradiometric assay.

③ Materials and Methods

IRMA using biotiniled Ab and radiolabelled streptavidin

Coated Ab   Ag to be dosed   Biotinylated Ab   Radiolabelled streptavidin
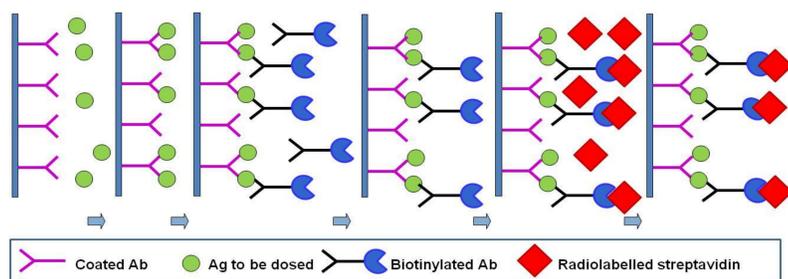
Fig. 1. The sample (volume until 4 mL) was incubated in the tube. After incubation, the whole sample was eliminated by several washes. Thereafter, an other antibody bound to biotin (detection antibody) was added in order to quantify the antigen present in the sample. After a second wash, the reaction was revealed by adding a Streptavidin tracer (^{125}I).

Reagents:

- Purified bovine PAG 67kDa was used as standard at concentrations ranging from 100 to 50,000 pg/mL.

- Highly purified immunoglobulin hp-Ig708 (purified from polyclonal antiserum raised against caprine PAG 55kDa+59kDa) was used as capture antibody (0.01 µg/tube).

- The hp-Ig727 (purified from polyclonal antiserum raised against purified boPAG67kDa) was used as detection antibody (1:8,000).

- Radiolabeled streptavidin (125I-Strep; 50,000 cpm/100 µL) was used to reveal the Ab-Ag-Ab-Biot complexes

Validation protocol:

a) <u>Minimum detection limit</u> (MDL) was calculated as the mean concentration minus twice SD of 10 duplicates of the zero (B0) standard.

b) <u>Reproducibility</u> was determined by calculating the intra- and inter-assay coefficients of variation (CV).

c) <u>Accuracy</u> was determined by adding increasing quantities of purified boPAG67kDa (10, 20, 40 and 80 pg) to bovine milk containing low PAG concentrations.

d) <u>Specificity</u> was tested regarding 16 different compounds including carbohydrates, aspartic proteinases, hormones and major milk proteins. These molecules were tested at dilutions ranging from 0.1 ng/mL to 100 µg/mL.

e) <u>Parallelism</u> was assessed by serially diluting pregnant cow milk containing relatively high PAG concentrations.

④ Results
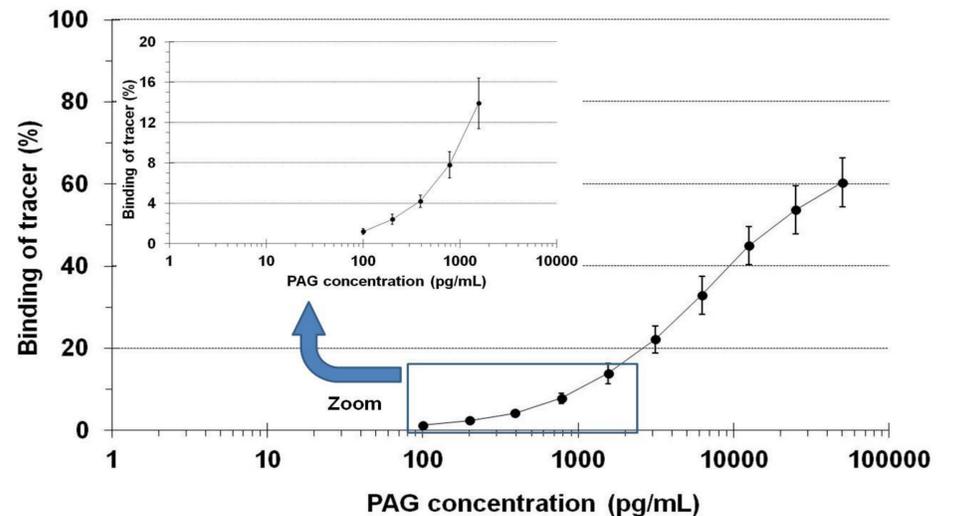
Mean standard curve of IRMA-PAG

Fig. 2. Binding ratio of eleven standard curves assayed by IRMA –PAG (mean ± SD). In the graphic inside (zoom) a focus was done on the std concentrations ranging from 100 to 1,560 pg/mL.

a) MDL was 4 pg/mL (more sensitive than RIA: 100 à 200 pg/mL)

b) Reproducibility:

- CV intra-assay (20 replicates in the same assay) : 13.7% (194.4 ± 26.6 pg/mL)
- CV inter-assay (10 different assays) : 15.8% (193.7 ± 30.6 pg/mL).

c) Accuracy:

Table 1. Recovery of boPAG added to a sample containing low PAG concentrations.

Sample	Initial concentration (pg/mL)	Qtty of PAG added (pg)	Theoretical concentration (pg/mL)	Observed concentration (pg/mL)	Recovery (%)
Milk	108.2	80	188.2	162.3	86.3
		40	148.2	135.5	91.5
		20	128.2	124.8	97.4
		10	118.2	123.9	104.8
		5	113.2	119.9	105.9

d) Specificity:

No cross reaction was observed with almost all tested compounds except lactoferin (2.4%), rennin (2.5%), porcine pepsinogen (2.3%) and porcine pepsin (5.2%) at 100 µg/mL (most of them at supra-physiological levels).
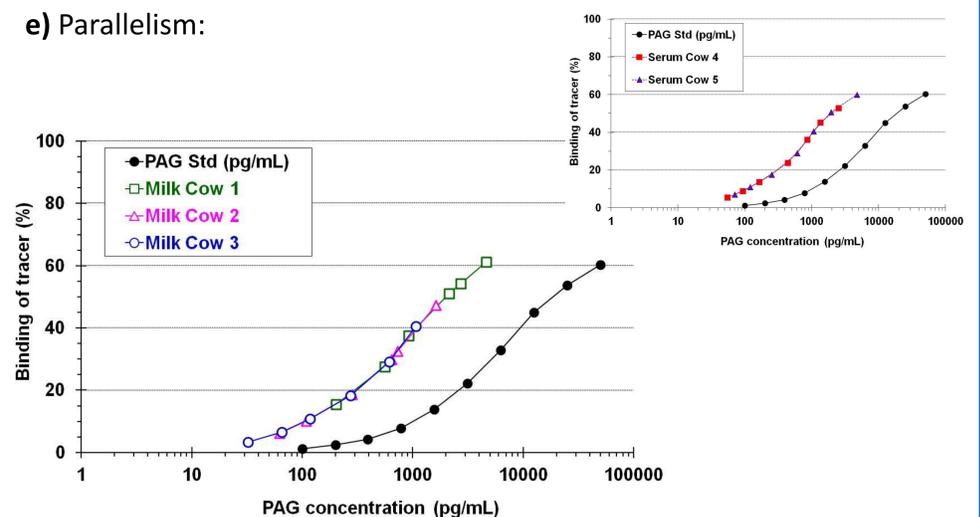
e) Parallelism:

Fig. 3. Parallelism between standard curves and serial dilutions of milk or serum from pregnant cows. Dilutions of bovine milk containing detectable PAG concentrations were parallel to standard curve.

⑤ Conclusions

• The new IRMA-PAG is highly sensitive, accurate, precise and reproducible for PAG measurements in bovine milk samples.
• Parallelism test reveals an excellent ability to quantify PAG concentrations in milk of dairy cattle.
• This new test can be an alternative method useful for pregnancy diagnosis and follow-up in bovine species without stress for sample collection.

Final OptiMIR Scientific and Expert Meeting: From milk analysis to advisory tools (Palais des Congrès, Namur, Belgium, 16-17 April 2015)

OPTIMIR - A PROJECT AIMING THE DEVELOPMENT OF NOVEL MID-INFRARED BASED MANAGEMENT TOOLS FOR DAIRY HERDS

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The dairy industry represents 13% of the turnover of the European food industry and north-west Europe produces 60% of the European milk. However, in 2014 the milk market has experienced a substantial fall in milk prices of some 50%. At these lower prices for milk the business of many milk producers is not sustainable. Due to recent advances, the mid-infrared (MIR) analysis of milk performed within milk recording (MR) promises more information than used traditionally. Beside the established MR parameters (protein, fat, lactose, and urea), the spectra could provide additional information on cows' status for a range of characteristics (*e.g.* fertility, health, energy balance, feeding and methane emission). Thus, the spectra routinely obtained from the MIR analysis of milk offer a possibility to develop novel, cost-effective tools which enable milk producers to improve the management of their dairy herds and in turn reduce the costs for milk production. The OptiMIR project aims to improve the sustainability of the dairy sector by developing and providing innovative, economical and standardized MIR-based tools for the management of dairy herds. Additionally, the project aims to create and promote a framework for the cross-border exchange of information and practices to enhance the service of milk recording organizations (MROs) in north-west Europe and to strengthen their competitiveness. In order to achieve these objectives, in 2011 the European OptiMIR project was officially launched for a 5-year-period. The INTERREG IV B funded OptiMIR project is a cooperation between three research centers, three universities, 11 MROs, and one laboratory from six north-western European countries. To accomplish the goals, the different processes from the identification of the priority areas to the development, validation, and implementation of the novel tools were outlined in three working packages comprising 10 actions. A common transnational database combines the phenotypic data of the cows and the MIR spectra from the European MR, which enables the detection of relevant phenotypic traits and their reflection in the MIR spectra. Additionally, a standardization has been installed among all instruments of the milk analyzing laboratories involved in the OptiMIR project to ensure a stable prediction over time and a correction of deviations. Due to the monthly standardization process the developed prediction equations can be used in routine on all instruments taking part in the standardization. Different models for the prediction of the pregnancy status, energy balance, the methane emission as well as the detection of ketosis and acidosis have been developed within OptiMIR. It is worth mentioning, that those models predict the status of the animal with different accuracies and that progress on development and implementation differs between the models. Some of the tools developed within OptiMIR are already used in the field and provide dairy farmers with useful information *e.g.* about the health status or methane emission of a certain cow. The OptiMIR project also resulted in a collaborative planning on an economic interest group formation of the participating MROs. The final OptiMIR scientific and expert meeting disseminates the results obtained through OptiMIR more detailed and also provides an overview of recent advances in the development of management tools for the dairy sector. Initial application of the MIR-based tools has shown their potential for providing dairy farmers with information to improve their herd management in a cost-effective way. However, further research and development is required to finish a greater extend of MIR-based tools so that north-western European stakeholders can acquire competitive advantage in the dairy sector.

Keywords. Dairy industry, livestock management, infrared spectrometry, research, Europe.