JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Rapid Time-Resolved Immunofluorometric Assay for the Measurement of Creatine Kinase in Serum and Whole Blood Samples

MIKA TUOMOLA,* JANI VAINIO,† AND TIMO LÖVGREN

Department of Biotechnology, University of Turku, FIN-20520 Turku, Finland

A rapid and simple immunochemical method was developed for the assessment of the creatine kinase (MM) isoenzyme [CK(MM)], a protein marker linked with animal welfare and meat quality. The one-step time-resolved immunofluorometric assay produced quantitative results from serum or whole blood samples in 20 min. The analytical limit of detection (mean + 2*s*) for the immunoassay was 17 ng/mL ($n = 6$), and the functional limit of detection for the analysis of porcine whole blood samples was 426 ng/mL ($n = 24$). The working range of the method was linear up to 50 μg/mL, and the within-assay precision varied between 2.1 and 10.9%. The analysis of porcine serum samples showed that the results from the immunoassay method and colorimetric CK enzyme activity determination were highly correlated ($r^2 = 0.965$, $n = 17$, $p < 0.001$). The practicability of the assay was demonstrated by the analysis of 300 porcine whole blood samples in a slaughterhouse environment.

KEYWORDS: Time-resolved fluorescence; immunoassay; creatine kinase; stress

INTRODUCTION

Animal welfare is a major issue in modern meat production, and the developments in housing and management practice of farm animals under intensive systems have to be balanced by concern for animal well-being. Transport and slaughter of farm animals are particularly important areas from a welfare point of view. In addition to ethical issues, welfare problems also cause economic losses through bruising, stress-induced meat quality problems, and even death during transport and lairage (*1*).

Some physiological responses can be used to assess the amount of effort animals must make to cope with the associated stresses. Physical activity and muscle damage change the permeability of mammalian muscle tissue, and increase the levels of certain tissue-specific enzymes, such as creatine kinase, in the blood (*2*). These enzymes can be used in animal production as markers of physical stress conditions that are directly linked with animal welfare and final meat quality. Creatine kinase (CK, EC 2.7.3.2) catalyzes the reversible reaction where creatine phosphate and ADP are converted to ATP and creatine (*3*). Cytoplasmic isoenzymes of creatine kinase are dimers composed of M and B subunits. CK(MM) is found in skeletal muscle and CK(BB) largely in brain, while the hybrid dimer, CK(MB), is located almost exclusively in heart. Blood CK measurements have frequently been used for the evaluation of stress or stress susceptibility in pigs (*4*−*9*) and cattle (*10*−*12*).

Analytical methods used in the past studies have been based on the measurement of the catalytic activity of CK (*13*−*15*). The methods have proven to be useful, but the samples have always needed some pretreatment which has slowed the test throughput. In addition, the measurement range has been limited, and the methods have had limitations common with many other enzymatic assays, such as nonspecificity with respect to isoenzymes, failure to detect inactive forms, and possible interference by inhibitors. The current paper describes a new tool for the analysis of CK(MM) that overcomes these problems by measuring the mass concentration instead of the catalytic activity of the enzyme. The method is based on the use of europium chelate labels and time-resolved fluorometry (TRF), and the technology allows the development of immunoassays that offer very high sensitivity and a wide dynamic range (for a review, see ref *16*). We have used TRF in the direct analysis of porcine serum samples with the dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) (*17*). However, the application for whole blood samples has been restricted due to the fact that the lanthanides in these kinds of tracers may dissociate from their chelates in the presence of other chelating compounds (e.g., EDTA). This has been solved in the current study by using intrinsically fluorescent and stable lanthanide chelates (*18*) as labels. The resulting simple one-step immunofluorometric assay produces quantitative results from whole blood samples in 20 min with a good sensitivity, reproducibility, and measurement range.

MATERIALS AND METHODS

Reagents. The fluorescent Eu chelate of [2,2′,2″,2‴-({4-[(4-isothiocyanatophenyl)ethynyl]pyridine-2,6-diyl}bis(methylenenitrilo))tetrakis-(acetato)] europium(III), biotin isothiocyanate (BITC), and streptavidin-coated microtitration plates were obtained from Innotrac Diagnostics Oy (Turku, Finland). The monoclonal anti-human CK(MM) antibody, affinity-purified goat anti-human CK(MM) antibody, and purified human hCK(MM) isoenzyme were supplied by Biospacific (Emeryville,

* To whom correspondence should be addressed. Telephone: +358 2 333 8089. Fax: +358 2 333 8050. E-mail: mika.tuomola@utu.fi.

† Present address: BioTie Therapies Corp., FIN-20520 Turku, Finland.

10.1021/jf025629v CCC: $22.00
Published on Web 09/18/2002

CA). Bovine serum albumin (BSA) was supplied by Intergen (Purchase, NY). The assay buffer used throughout consisted of 50 mM Tris-HCl buffer (pH 7.75) containing 0.9% NaCl, 0.05% NaN_3, 0.5% BSA, 0.01% Tween 40, 0.05% bovine γ-globulin, 20 μM diethylenetriaminepentaacetic acid (for binding possible free lanthanide ions), and 20 μg/mL Cherry Red (for making the pipetting easier). The wash solution consisted of 5 mM Tris-HCl buffer (pH 7.75) containing 0.9% NaCl and 0.05% Tween 20.

Samples. Porcine blood samples were taken at a commercial abattoir on one day during bleeding. Whole blood samples ($n = 300$) were collected in EDTA tubes. Additional samples ($n = 17$) were taken in empty tubes, and the blood was allowed to clot for 1−2 h and serum isolated by centrifugation. All samples were stored at −20 °C.

Purification of the Porcine Creatine Kinase MM Isoenzyme [pCK(MM)]. A sample of porcine *longissimus dorsi* muscle (40−50 g) was rinsed with saline, trimmed to remove fat and connective tissue, and processed at 4 °C as described by Grace and Roberts (*19*). In brief, the tissue was homogenized and precipitated twice with ethanol and recovered in 50 mM Tris-HCl buffer (pH 8.0) with 5 mM 2-mercaptoethanol. A sample of the extract (10−15 mL) was applied to a DEAE-Sepharose Fast Flow column (1.5 cm × 30 cm, 45 mL, packed in the lab) (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with 50 mM Tris-HCl buffer (pH 8.0) with 5 mM 2-mercaptoethanol at 4 °C. The column was eluted with a linear sodium chloride gradient (from 0 to 500 mM) in the same buffer at a flow rate of 1 mL/min and monitored by UV absorbance at 254 nm. Fractions (1 mL) were collected and tested with an immunofluorometric assay. Immunoreactive fractions from the two emerging peaks were pooled separately, and the protein concentration was measured by the Bradford method (Bio-Rad Protein Assay Kit, Bio-Rad Laboratories, Hercules, CA). The protein purity was assessed by polyacrylamide gel electrophoresis (SDS−PAGE) by using a PhastGel 8−25 gradient gel run and stained with the silver staining kit in a PhastSystem (Amersham Pharmacia Biotech).

Labeling of the CK(MM) Antibody. The labeling of the polyclonal antibody was performed with a 100-fold molar excess of label reagent in 50 mM borate buffer (pH 8.5) at 4 °C in an overnight incubation. The labeled antibody was separated from the excess free label on a Superdex 200 HR 10/30 gel filtration column (Amersham Pharmacia Biotech), equilibrated, and run with 50 mM Tris-HCl (pH 7.75) containing 0.15 M NaCl and 15 mM NaN_3. The column was operated at room temperature with a flow rate of 15 mL/h. Bovine serum albumin was added to a final concentration of 0.1%, and the solution was filtered through a 0.22 μm pore-size filter (Millipore Corp.) before being stored at 4 °C.

Biotinylation of the CK(MM) Antibody. Biotinylation of the monoclonal CK(MM) antibody was performed in 50 mM sodium carbonate buffer (pH 9.8) containing a 100-fold molar excess of BITC dissolved in dimethylformamide. This mixture was incubated for 4 h at room temperature. The biotinylated antibody was separated from the excess reagent by passing the mixture through NAP-5 and NAP-10 columns (both from Amersham Pharmacia Biotech) with 50 mM Tris-HCl (pH 7.75) containing 0.15 M NaCl and 15 mM NaN_3 as the eluent. BSA was added as a stabilizer to the purified conjugates at a concentration of 0.1%.

One-Step Time-Resolved Immunofluorometric Assay (TR-IFMA) for CK(MM). The microtitration well plates were prepared in advance by adding 30 μL (200 ng) of the biotinylated monoclonal antibody in assay buffer to streptavidin-coated microtitration wells. The plates were incubated for 30 min at room temperature and washed four times with wash solution. The plates were stored at 4 °C before being used. The assay was started by adding hCK(MM) standards and porcine whole blood or serum samples (5 μL, prediluted 1:10 with assay buffer) and the Eu-labeled tracer antibody (25 μL) into the wells. The wells were incubated for 15 min at 37 °C in an iEMS incubator/shaker (Labsystems Oy, Helsinki, Finland) and washed four times. A fluorescence enhancement solution (200 μL), containing 50 mM glycine-NaOH (pH 10), 1.75 M NaSCN, 5 mM Na_2CO_3, 1 M NaCl, 5% glycerol, and 20% 1-propanol, was added to the wells, and they were shaken for 3 min before the signal was measured with a DELFIA 1234 time-resolved fluorometer (PerkinElmer Life Sciences, Turku, Finland).

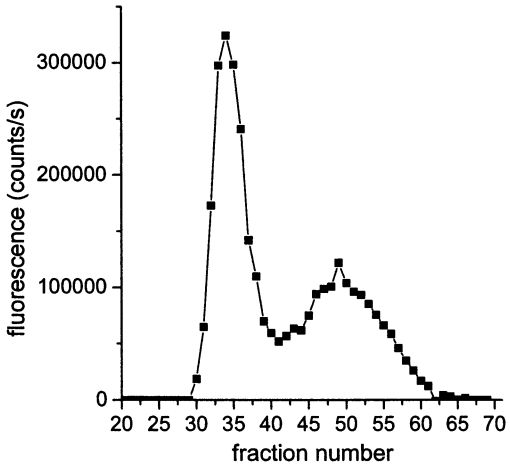

Figure 1. Elution profile of porcine skeletal CK(MM) on a DEAE-Sepharose Fast Flow column based on immunoreactivity measurement.

Measurement of CK Enzymatic Activity. The creatine kinase activity of porcine serum samples was analyzed with enzymatic Sigma procedure UV-47 (Sigma Diagnostics, St. Louis, MO) according to the manufacturer's instructions.

RESULTS AND DISCUSSION

To our knowledge, no antibodies have been developed against porcine CK(MM) up to date. However, the level of primary amino acid sequence homology of human and mammalian CK(MM) is >90%, so the probability of finding common epitopes from them is relatively high. We tested this hypothesis in the preliminary work by evaluating the binding properties of a number of commercially available anti-human CK(MM) antibodies in a TR-IFMA system by using crude preparations of porcine and bovine CK(MM) or serum samples as antigens. Almost all the monoclonal antibodies bound only the human antigen, but the polyclonal antibody and one monoclonal antibody also recognized porcine and bovine CK(MM), which proves that some epitopes are conserved between the species. These two antibodies were used for the development of the immunometric assay described here.

The ratios of different CK isoforms are slightly variable between different animal species, but especially in pigs, the CK(MM) isoenzyme comprises close to 100% of the total CK found in the skeletal muscles (*4*, *20*). The elution pattern of pCK(MM) in anion exchange chromatography is shown in **Figure 1**.

The fractions from the first peak had intense immunoreactivity, and electrophoresis of the pooled fractions on an SDS−polyacrylamide gel showed a single protein band with mobility identical to that of the hCK(MM) standard. The second peak probably contained denatured forms of CK(MM). The pooled fractions from this peak contained a larger amount of protein; however, the immunoreactivity was low, and SDS−PAGE revealed the presence of multiple bands. The purified pCK was used in the method validation for recovery estimation, but the assay was calibrated using hCK standards because the immunoreactivity of the pCK preparation was less stable and pure hCK was readily available from commercial sources. The assay response was, however, greater for the native hCK(MM) antigen. The reactivity of pCK(MM) was less than 10% when compared with that of the hCK(MM) standard, and the results of the assays are therefore expressed here as human CK(MM) equivalents [heCK(MM)].

The reagents and incubation conditions of the assay were optimized essentially to bring the bioaffinity reaction quickly

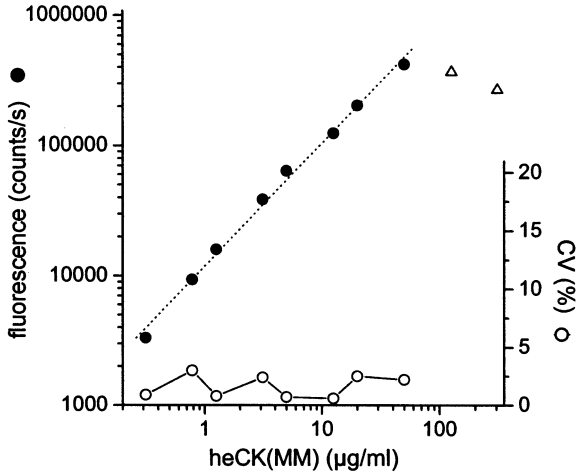

Figure 2. Standard curve (●) and precision profile (○) of the CK(MM) TR-IFMA method. Concentrations were measured against a human antigen standard, and the results are expressed as human CK equivalents [heCK(MM)]. Regression data: $\log(y) = \log(x) \times 0.946 + 4.074$, $r^2 = 0.995$, $n = 4$, and $p < 0.001$. The results of the hook-effect study outside the method working range are shown with empty triangles.

close to the equilibrium. The assay kinetics were tested with pCK(MM) (15.0 μg/mL) and hCK(MM) (20.0 μg/mL) standards and porcine whole blood sample containing endogenous CK(MM) (11.5 μg/mL). After 10 min of reaction time, the assay response for these samples was already 94.1−95.8% compared with the results obtained after incubation for 30 min. The final incubation time was set to 15 min to ensure good assay reproducibility. The standard curve of the CK(MM) assay is shown in **Figure 2**. The analytical limit of detection was evaluated by repeated analysis of the zero calibrator ($n = 6$) and gave a concentration of 17 ng/mL (mean + 2*s*). The functional limit of detection was determined in a similar manner by analyzing porcine whole blood samples giving very low responses in the assay ($n = 24$) and equaled 426 ng/mL. The value is, however, most likely an overestimation because the normal basic CK level is higher in pigs than, for example, in humans or dogs (*21*) and true zero-level samples cannot be obtained. The quantitation limit in porcine whole blood samples was set to 470 ng/mL (mean + 3*s*), and the recovery of this concentration of pCK(MM) added to samples with no measurable CK(MM) was 98% with a variation coefficient of 10% ($n = 15$).

The assay working range was linear up to 50 μg/mL, and the precision varied between 0.6 and 3.0%. The effect of very high concentrations (125.8 and 306.2 μg/mL) was also studied because the response of immunometric one-step assays may start to decrease at high analyte levels. This high-dose hook effect happens when the binding capacity of the capture antibody is exceeded and part of the analyte and labeled antibody is washed away. The tested concentrations are unlikely to occur in real samples, but the results plotted in **Figure 2** show that the decrease in signal is so small that the clinical implications would remain unaffected. The accuracy of the method was determined by fortifying whole blood samples with different levels of pCK(MM). The results in **Table 1** show that the sample dilution provided sufficient recovery of the analyte with an average of 91.4%. Assay repeatability was determined by the repeated analysis of three porcine blood samples containing different levels of CK(MM) (**Table 2**), and the within-assay precision varied between 2.1 and 10.9%.

The immunochemical analysis of the CK(MM) mass concentration was compared against CK enzyme activity determination by analyzing porcine serum samples. As shown in **Figure 3**, the results from the two methods were highly correlated ($r^2 = 0.965$, $p < 0.001$).

Table 1. Accuracy of the TR-IFMA Method ($n = 4$)

sample	pCK(MM) added, ng/mL [heCK(MM)]	% recovery
1	5960	88.2
2	14810	92.0
3	29620	95.4
4	49750	89.9

Table 2. Precision of the TR-IFMA Method

sample	mean CK(MM), ng/mL [heCK(MM)]	interassay cv% ($n = 12$)	intra-assay cv% ($n = 8$)
1	990	13.1	10.9
2	8170	3.8	2.1
3	14550	7.0	3.5

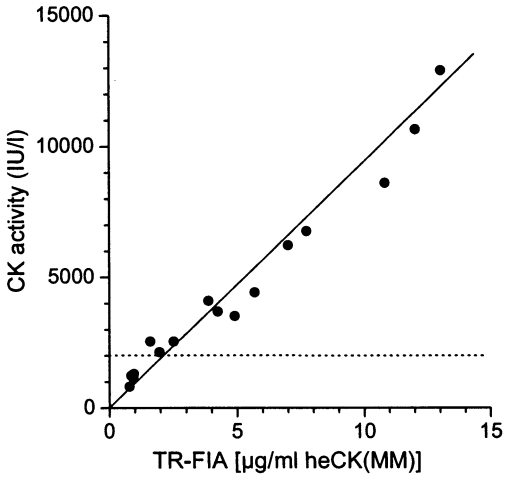

Figure 3. Mass concentration vs enzyme activity of CK(MM) in porcine serum samples ($n = 17$). Immunoassay results expressed as human CK equivalents [heCK(MM)]. Linear regression data: $y = 942.7x + 11.7$, $r^2 = 0.965$, and $p < 0.001$. The dotted line demonstrates the upper limit of the activity method after which the samples need to be diluted further.

It is evident that the practical working range of the immunoassay is superior to that of the activity method. The dotted line in **Figure 3** demonstrates the upper limit of the colorimetric method, after which the samples have to be reanalyzed by using a higher dilution factor. Another major advantage of the current TR-IFMA method is the fact that it is not disturbed by the presence of red blood cells or strongly chelating polycarboxylic acids, such as EDTA which is commonly used as an anticoagulant. The use of unprocessed whole blood samples makes the total method very simple and enables fast sample throughput. Analyte concentrations are of course lower in whole blood samples than in serum or plasma samples due to the volume taken by red blood cells. The difference can be eliminated almost completely if the results from whole blood samples are corrected by the corresponding hematocrit values, but normal individual variations in the volume of the red blood cells, however, seem to have a fairly insignificant effect on the results (*22*). This effect was not investigated in the current study, but it is worth mentioning that CK(MM) levels in bovine serum and whole blood samples were very highly correlated ($r^2 = 0.976$, $n = 20$, $p < 0.001$, $y = 0.600x + 0.092$) when analyzed by the current TR-IFMA method (unpublished results).

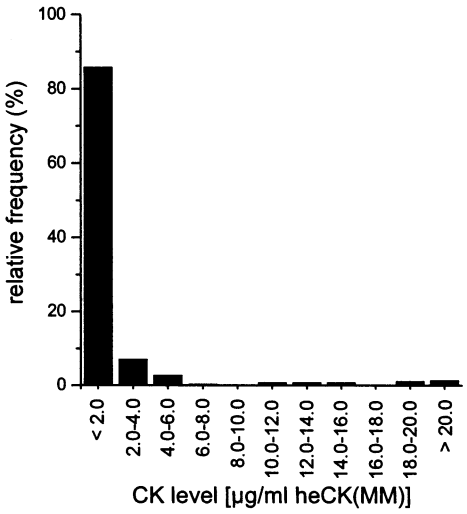

Figure 4. Distribution of CK(MM) levels in porcine whole blood samples taken consecutively from the production line of a commercial abattoir ($n = 300$). Immunoassay results expressed as human CK equivalents [heCK(MM)].

The method was applied to the analysis of 300 porcine whole blood samples at a commercial abattoir. The samples were taken from normal slaughter pigs at the production line during bleeding, and they were analyzed immediately. The results were available less than 30 min after the slaughter. The frequency distribution of CK(MM) levels in the sample population is given in **Figure 4** and shows that the concentrations were low in the majority of the samples. Tentative cutoff values of 2.0, 4.0, and 6.0 μg/mL heCK(MM) were exceeded by 14.3, 7.3, and 4.7% of the pigs, respectively. This limited study demonstrated that the screening of elevated levels of CK(MM) could be performed very efficiently with the method described herein. The immunochemical assay provides a very efficient and user-friendly alternative for the traditional enzyme activity determination and a useful tool for the estimation of animal stress at various stages of animal production. The immunoassay technology based on the use of time-resolved fluorescence is also well-suited for on-line analysis because the one-step assay is easy to automate and it allows rapid quantitative assays to be carried out from difficult sample matrices such as whole blood samples.

ACKNOWLEDGMENT

We thank Mrs. Eeva-Riitta Wirta for her help in the collection of the samples.

LITERATURE CITED

(1) Grandin, T. Introduction: management and economic factors of handling and transport. In *Livestock Handling and Transport*; Grandin, T., Ed.; CAB International: Wallingford, U.K., 1993; pp 1−9.

(2) Warnants, N.; Eeckhout, W.; Boucqué, Ch. V. Serum puryvate kinase and its relation to stress susceptibility in pigs. *J. Anim. Breed. Genet.* **1993**, *110*, 357−362.

(3) Eppenberger, H. M.; Eppenberger, M.; Richterich, R.; Aebi, H. The ontogeny of creatine kinase isoenzymes. *Dev. Biol.* **1964**, *10*, 1−16.

(4) Hallberg, J. W.; Topel, D. G.; Christian, L. L. Creatine phosphokinase isoenzymes in stress-susceptible and stress-resistant pigs. *J. Anim. Sci.* **1979**, *49*, 1464−1469.

(5) Honkavaara, M. Influence of porcine stress on blood composition and early post-mortem meat quality in pigs of different halothane genotypes. *Meat Sci.* **1988**, *24*, 21−29.

(6) Barton Gade, P.; Christensen, L. Effect of different stocking densities during transport on welfare and meat quality in Danish slaughter pigs. *Meat Sci.* **1998**, *48*, 237−247.

(7) Warriss, P. D.; Brown, S. N.; Adams, S. J. M.; Corlett, I. K. Relationships between subjective and objective assessments of stress at slaughter and meat quality in pigs. *Meat Sci.* **1994**, *38*, 329−340.

(8) Warriss, P. D.; Brown, S. N.; Knowles, T. G.; Edwards, J. E.; Kettlewell, P. J.; Guise, H. J. The effect of stocking density in transit on the carcass quality and welfare of slaughter pigs: 2. Results from the analysis of blood and meat samples. *Meat Sci.* **1998**, *50*, 447−456.

(9) Gispert, M.; Faucitano, L.; Oliver, M. A.; Guàrdia, M. D.; Coll, C.; Siggens, K.; Harvey, K.; Diestre, A. A survey of pre-slaughter conditions, halothane gene frequency, and carcass and meat quality in five Spanish pig commercial abattoirs. *Meat Sci.* **2000**, *55*, 97−106.

(10) Warriss, P. D.; Kestin, S. C.; Brown, S. N.; Wilkins, L. J. The time required for recovery from mixing stress in young bulls and the prevention of dark cutting beef. *Meat Sci.* **1984**, *10*, 53−68.

(11) Tarrant, P. V.; Kenny, F. J.; Harrington, D. The effect of stocking density during 4 hour transport to slaughter on behaviour, blood constituents and carcass bruising in Friesian steers. *Meat Sci.* **1988**, *24*, 209−222.

(12) Mohan Raj, A. B.; Moss, B. W.; Rice, D. A.; Kilpatrick, D. J.; McCaughey, W. J.; McLauchlan, W. Effect of mixing male sex types of cattle on their meat quality and stress-related parameters. *Meat Sci.* **1992**, *32*, 367−386.

(13) Rosalki, S. B. Improved procedure for creatine phosphokinase determination. *J. Lab. Clin. Med.* **1967**, *79*, 696−705.

(14) Szasz, G.; Gruber, W.; Bernt, E. Creatine kinase in serum: 1. Determination of optimum reaction conditions. *Clin. Chem.* **1976**, *22*, 650−656.

(15) Scheuerbrandt, G.; Lundin, A.; Lövgren, T.; Mortier, W. Screening for Duchenne muscular dystrophy: An improved screening test for creatine kinase and its application in an infant screening program. *Muscle Nerve* **1986**, *9*, 11−23.

(16) Lövgren, T.; Pettersson, P. Time-resolved fluoroimmunoassay, advantages and limitations. In *Luminescence immunoassay and molecular applications*; Van Dyke, K., Van Dyke, R., Eds.; CRC Press: Boca Raton, FL, 2000; pp 233−253.

(17) Tuomola, M.; Harpio, R.; Knuuttila, P.; Mikola, H.; Lövgren, T. Time-resolved fluoroimmunoassay for the measurement of androstenone in porcine serum and fat samples. *J. Agric. Food Chem.* **1997**, *45*, 3529−3534.

(18) Mitrunen, K.; Pettersson, K.; Piironen, T.; Björk, T.; Lilja, H.; Lövgren, T. Dual-label one-step immunoassay for simultaneous measurement of free and total prostate-specific antigen concentrations and ratios in serum. *Clin. Chem.* **1995**, *41*, 1115−1120.

(19) Grace, A.; Roberts, R. Improved procedures for purification of human and canine creatine kinase isoenzymes. *Clin. Chim. Acta* **1982**, *123*, 59−71.

(20) Fredericks, S.; Merton, G. K.; Lerena, M. J.; Heinig, P.; Carter, N. D.; Holt, D. W. Cardiac troponins and creatine kinase content of striated muscle in common laboratory animals. *Clin. Chim. Acta* **2001**, *304*, 65−74.

(21) Doizé, F.; Deroth, L. Identification by isoelectric focusing of creatine kinase-MM isoforms in the plasma and striated muscle of pigs. *Vet. Res. Commun.* **1991**, *15*, 279−284.

(22) Pettersson, K.; Katajamäki, T.; Irjala, K.; Leppanen, V.; Majamaa-Voltti, K.; Laitinen, P. Time-resolved fluorometry (TRF)-based immunoassay concept for rapid and quantitative determination of biochemical myocardial infarction markers from whole blood, serum and plasma. *Luminescence* **2000**, *15*, 399−407.

Received for review April 26, 2002. Revised manuscript received August 15, 2002. Accepted August 16, 2002.

JF025629V