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Immunological detection of osteocalcin in meat and bone meal: a novel heat stable marker for the investigation of illegal feed adulteration

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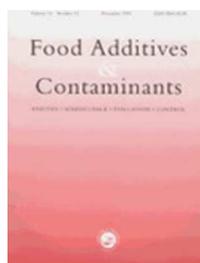
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Immunological detection of osteocalcin in meat and bone meal: a novel heat stable marker for the investigation of illegal feed adulteration

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Abstract:	A sandwich ELISA was developed for the detection of bovine meat and bone meal (BMBM) in feed, based on polyclonal rabbit antibodies raised against the synthetic N-terminal amino acid sequence 1–9 (YLDHWLGAP) of bovine osteocalcin. To set up a sandwich ELISA pair, a commercial mouse monoclonal capture antibody binding to a highly conserved epitope in the mid-fragment of the peptide was employed. It is shown that the bone marker osteocalcin is immunologically well detectable in BMBM extracts obtained by a simple EDTA based procedure even in a sample heated up to 145 °C. Furthermore a genus-specific restriction of the major specificity to cattle and horse was possible. The observed bi-specificity is consistent with theoretical predictions. The assay sensitivity with bovine osteocalcin of 1 ng was sufficient to enable the detection of 0.1% BMBM in compound plant feed or fish meal, for which no cross reaction was observed. In general the quantification of osteocalcin in extracts is possible using a standard curve procedure with pure bovine osteocalcin.

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5 2 **novel heat stable marker for the investigation of illegal feed**
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8 3 **adulteration**
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28
29 13 **Abstract**
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33 15 A sandwich ELISA was developed for the detection of bovine meat and bone meal (BMBM)
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35 16 in feed, based on polyclonal rabbit antibodies raised against the synthetic N-terminal amino
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37 17 acid sequence 1–9 (YLDHWLGAP) of bovine osteocalcin. To set up a sandwich ELISA pair,
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39 18 a commercial mouse monoclonal capture antibody binding to a highly conserved epitope in
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47 22 the major specificity to cattle and horse was possible. The observed bi-specificity is
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49 23 consistent with theoretical predictions. The assay sensitivity with bovine osteocalcin of 1 ng
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51 24 was sufficient to enable the detection of 0.1% BMBM in compound plant feed or fish meal, for
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53 25 which no cross reaction was observed. In general the quantification of osteocalcin in extracts
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55 26 is possible using a standard curve procedure with pure bovine osteocalcin.
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28 **KEYWORDS:** Osteocalcin, Meat-and-Bone-Meal (MBM), Bovine spongiform encephalopathy
29 (BSE), Animal feed, Processed animal proteins (PAPs), ELISA, Species identification

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30 Introduction

31 Feeding meat and bone meal (MBM) to farmed animals is prohibited in Europe by Regulation
32 (EC) No 999/2001. In addition, an overarching species-to-species feed ban has been laid
33 down by Regulation (EC) No 1069/2009. The only exemption from the strict MBM ban is fish
34 meal, which has been allowed for pig and fowl feeding and recently also for calves in milk
35 replacing feed mixtures (Regulation (EC) No 999/2001). According to the recitals of the
36 above mentioned Regulation it might be assumed that the feed ban for ruminants will not be
37 lifted in the near future whereas the prohibition of processed animal proteins (PAP) feeding
38 to other animals than ruminants might be relaxed as far as analytical tools for species
39 identification are available.

40
41 The official method for the detection of MBM in feeding stuff is based on classical light
42 microscopy (Regulation (EC) No 152/2009). A second test may also be carried out using
43 variant or alternative methods, in order to improve the detection of certain types of animal
44 constituents or to further specify the origin of the animal constituents. The microscopic-based
45 method reliably detects heat- and pressure-stable particles of animal origin like bone
46 fragments, teeth, hair, gills or scales which are resisting the legally required MBM treatment
47 of 133 °C for 20 min at 300 kPa (Liu et al. 2011). However, by applying the microscope
48 approach it is not possible to distinguish between species or genus in samples containing
49 PAPs. Thus, up to now the implemented species-to-species ban cannot be controlled.
50 Furthermore, the microscopic investigation depends solely on individual skills and allows an
51 estimation but not quantification of the MBM content in a feed sample. Therefore - and
52 against the background of recently discussed thresholds and a further lifting of the feed ban
53 in the European Union (COMMISSION STAFF WORKING DOCUMENT 2010) - there is an
54 urgent need for alternative methods which might overcome present drawbacks to enable
55 legally consistent feed survey and law enforcement.

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3 57 The immunological detection of PAPs is for long used as an alternative to microscopy.
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5 58 Immunoassays can be performed in any laboratory independently from sophisticated
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7 59 equipment or particularly trained staff. Antibody based assays are convertible into rapid
8
9 60 formats like lateral flow assays (dip stick tests) representing most convenient field methods
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11 61 for rapid screening purposes. Ruminant or pork specific antibodies binding to PAPs, so far
12
13 62 have been raised either directly against bovine meat and bone meal (MBM) (Muldoon et al.
14
15 63 2004; Kim et al. 2004), mixtures of muscle proteins (Kim et al. 2005; Chen et al. 1998;
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17 64 Ansfield 1994), purified troponin I (Chen et al. 2002) or *h*-caldesmon (Kim et al. 2004),
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19 65 isolated from skeletal or smooth muscle of the bovine intestine, respectively. Four
20
21 66 commercial PAP detecting test kits (two dip stick tests and two ELISA) based on antibodies
22
23 67 binding to the described targets have been repeatedly evaluated in studies conducted by
24
25 68 official institutions in the USA or Europe. All of them revealed different shortcomings in terms
26
27 69 of specificity, selectivity or sensitivity (Fumière et al. 2009). Moreover, from the viewpoint of
28
29 70 validation, it is not satisfying that neither the kit manuals nor accompanying scientific
30
31 71 publications disclose the precise nature of the targeted epitopes, neither amino acid
32
33 72 sequences nor conformational molecular reaction sites, involved in the antigen-antibody
34
35 73 binding. As a consequence, potential cross reactivity cannot be deduced *in silico* and the
36
37 74 likelihood to reproduce immunization results obtained with changing or undefined antigen
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39 75 mixtures is low.

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43 77 The polymerase chain reaction (PCR) is another alternative approach to identify animal
44
45 78 species-specific components in feedstuffs. PCR is highly-specific and is amplifying target
46
47 79 DNA sequences in an exponential way. However PCR is not tissue-specific due to the fact
48
49 80 that the DNA of an individual organism does not differ between different tissues. Therefore it
50
51 81 is not possible to distinguish if the detected DNA is derived from the use of MBM or from
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53 82 allowed animal products such as milk or egg protein.
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3 84 In the present study we employed defined target sequences of bovine osteocalcin, consisting
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5 85 of the amino acid sequence 1-9 and the epitope 17 of the peptide, to establish an ELISA for
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7 86 the purpose of MBM detection in feed. Osteocalcin is a small extracellular bone matrix
8
9 87 protein produced by osteoblasts and a common constituent in calcified bone tissue all over
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11 88 the vertebrate group including bone fish (Hauschka et al. 1989; Nishimoto et al. 2003; Laizé
12
13 89 et al. 2005). The mature peptide of approx. 5800-5900 Da size is composed of 45 – 49
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15 90 amino acids. Up to 90 % of osteocalcin is secreted inside the bone tissue where it is, apart
16
17 91 from collagen, the most abundant bone protein accounting up to 2 mg/g bone matrix or 1 – 5
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19 92 % (w/w) of the total bone protein, respectively 10 – 20 % (w/w) of the non-collagenous bone
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21 93 proteins (Hauschka et al. 1989). A minor share of osteocalcin (10 - 40 %) is circulating in the
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23 94 blood where it is - once detached from the bone mineral - quickly degraded in kidney and
24
25 95 liver (Lee et al. 2000). The function of osteocalcin is not completely understood but the
26
27 96 molecule seems to play a role in the molecular organisation of the hydroxyapatite skeleton as
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29 97 well as in bone turnover (Dowd et al. 2003; Ducy et al. 1996). Three glutamic acid residues
30
31 98 are located at amino acid position 17, 21 and 24, constituting a highly conserved motif of the
32
33 99 peptide. These glutamic acids are transformed into γ -carboxyglutamic acids in a post-
34
35 100 translational carboxylation step and function as precipitation sites for Ca^{2+} ions. In the
36
37 101 presence of calcium the molecule forms a compact structure comprising of two α -helical
38
39 102 regions and a hydrophobic core near the C-terminus (Dowd et al. 2003). Both, N- and C-
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41 103 terminal sequences of osteocalcin reveal species specific differences (Figure 1).
42
43 104 Osteocalcin is known to be heat stable. Human or bovine bone osteocalcin which was
44
45 105 extracted at 120°C overnight, lyophilized and decarboxylated at 110 °C for five hours, still
46
47 106 retained its substrate specificity towards enzymatic vitamin K-dependent carboxylase
48
49 107 (Vermeer et al. 1984). Osteocalcin could be identified in 75.000-year-old Neanderthal bone
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51 108 by MALDI-TOF/TOF-MS (Nielsen-Marsh et al. 2005) and in fossil bones and teeth by
52
53 109 immunoassay-based investigation (Ulrich et al. 1987; Collins et al. 2000; Muyzer et al. 1992).
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55 110 We recently demonstrated that osteocalcin can be identified and differentiated in MBM of
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3 111 bovine and pig origin using high-resolution (MALDI/TOF-MS and HR-ESI Q/TOF) mass
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5 112 spectrometry (Balizs et al. 2011).
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9 114 The aim of the present study was to investigate i) if osteocalcin is still immunologically
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11 115 detectable in MBM treated according to the legal requirements, ii) how far a discrimination of
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13 116 farmed animals, especially cattle in feed, is possible and iii) to evaluate if a selectivity in an
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15 117 ELISA down to 0.1 % MBM either in compound feed or fish meal would be achievable.
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19 119 **Materials and Methods**

21 120 *Materials*

22
23 121 Pure bovine osteocalcin (OC) was purchased from Merck Calbiochem (Merck, Darmstadt,
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25 122 Germany). Bone from farmed animals and pasteurized egg- and milk powder were
26
27 123 purchased from local retailer in Berlin. 100 % pure bovine meat and bone meal (BMBM),
28
29 124 treated 145 °C, with a bone content of approx. 80 %, as well as 100 % sheep meat and bone
30
31 125 meal (OMBM) were kindly submitted by the European Reference Laboratory for Animal
32
33 126 Proteins EU-RL AP, Gembloux, Belgium. From the same source a spiked 0.1 % BMBM
34
35 127 sample in plant feed (Plant Feed-0.1 % BMBM), serving as reference and training material
36
37 128 for microscopists, was obtained. This material was purity checked by classical microscopy
38
39 129 and PCR. Based on the results the EU-RL AP specified the material as a mix of four pure
40
41 130 bovine meat and bone meals from different heat treatments with a bone content of ~ 54 %.
42
43 131 On request, the EU-RL AP also kindly made available a sample of the non-contaminated
44
45 132 plant feed background (Plant Feed-0 % BMBM), used for spiking consisting of a classical
46
47 133 feed mix containing wheat, tapioca, soybean, rapeseed, palm kernel and barley meals, beet
48
49 134 pulp, molasses, bakery by-products, vegetable fat, limestone, salts and vitamins. A
50
51 135 commercial dog feed BMBM (100 % cattle according to the label) was purchased from
52
53 136 PerNaturam V.O.F. (Aalten, The Netherlands). Fish meal (FM) and poultry meal (PM)
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56 137 produced under unknown processing parameters were obtained from diverse rendering
57
58 138 plants in Germany (SARIA Bio-Industries GmbH & Co., Selm, Germany). An anonymous
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3 139 sample of BMBM treated in accordance with legal requirements (133 °C) was obtained from
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5 140 the Department of Feed and Feed Additives of the Federal Institute for Risk Assessment,
6
7 141 Berlin. Pure pig meal from an anonymous source was kindly supplied by LUFA Nord-West
8
9 142 (Oldenburg, Germany). Samples containing 0.1, 1 and 10 % (v/v) BMBM (dog feed) extract
10
11 143 were prepared by mixing respective volumes of MBM extracts containing a comparable total
12
13 144 protein concentration.

145

146 *Sample preparation and protein extraction*

147 For the production of reference proteins, native bones from different species were cleaned
148 from any fat, meat residues and bone marrow and roughly trenched. Bone fragments were
149 degreased by Soxhlet extraction in a Soxtec™ 1045 apparatus (FOSS GmbH, Rellingen,
150 Germany) with boiling petroleum ether (Merck, Darmstadt, Germany). The defatted material
151 was transferred into steel cartridges, cooled down for 2-3 minutes with liquid nitrogen and
152 ground to yield a fine powder using a steel ball mill (TissueLyser Qiagen, Cat.No.85220,
153 Retsch GmbH, Haan, Germany) for 3 min and 30 Hz.

154 MBM, plant feed or bone powder was extracted by EDTA-extraction. All centrifugation steps
155 were performed at 15000 g, 10-20 min, 4 °C in a Heraeus Multifuge® 3S+/3SR+ (Thermo
156 Scientific, Schwerte, Germany). In brief, 2 g sample were extracted with 8 ml 0.5 M
157 Ammonium EDTA (pH 6.1) by stirring at 4 °C over night. The slurry was transferred into a
158 conical Falcon™ tube and centrifuged. The supernatant was transferred into ultrafiltration
159 tubes with an exclusion size of 3000 Da (Amicon® Ultra Centrifugal Filters Ultracel-3K,
160 Millipore, Ireland) and the retained protein fraction was washed with 2 ml phosphate buffered
161 saline (PBS, 10 mM NaCl, 10 mM KCl, 10 mM Na₂HPO₄, -NaH₂PO₄, pH 7.4) to exchange the
162 buffer.

163 The protein concentration was measured for all extracts in triplicates using a Bicinchoninic
164 Acid Protein Assay Kit (Sigma-Aldrich, Saint Louis, Missouri, USA) according to the
165 manufacturer's instruction. All extracts (Table 1) were aliquoted and stored at - 80 °C until
166 use.

167

168 *Antibodies*

169 A commercial mouse monoclonal antibody binding to the epitope aa17 of bovine osteocalcin
170 (OC4-30, Takara Bio Inc., Otsu/Shiga, Japan), specified to cross react with human, bovine,
171 rabbit, sheep, dog, monkey and goat but not rat or mouse, was used as animal specific
172 capture antibody in a two-side sandwich ELISA. Polyclonal antibodies (PAB) were raised in
173 two rabbits against the synthetic and LPH- (*Limulus polyphenus hemocyanin*) coupled
174 osteocalcin amino acid sequence OC 1-9 (YLDHWLGAP) by a service laboratory (Biogenes,
175 Berlin). The best performing serum in a pre-test against BSA-coupled amino acid sequence
176 (PAB OC1-9) was purified by affinity chromatography on CNBr-activated sepharose by the
177 service laboratory to isolate a monospecific IgG-fraction which was used in the sandwich
178 ELISA as secondary antibody. For this purpose PAB OC1-9 was conjugated with streptavidin
179 (PAB OC1-9-Strep) using a commercial kit according to the manufacturer's instruction
180 (Lightning-Link™ Streptavidin Conjugation Kit, Innova Biosciences, Cambridge, UK,
181 Technical Bulletin 1086, Release 02, June 2010).

182

183 *Sandwich ELISA*

184 Capture, secondary and detecting agent concentrations as well as the incubation time and
185 temperature were optimized in grid experiments. The final protocol was performed as follows.
186 Washing steps were repeated in general four times with 380 µl PBS-Tween 20 (154 mM
187 NaCl, 7.7 mM Na₂HPO₄, 2 mM NaH₂PO₄, 0.05% Tween 20, pH 7.4) using an automatic
188 ELISA Washer (Wellwash, Thermo Fisher Scientific). All incubation steps were carried out in
189 a volume of 100 µl/well. 1 µg/ml of the capture antibody (OC4-30) was bound to a microtiter
190 plate (Maxisorp, Nunc™, Denmark) for 48h at 4°C. The plate was washed and then blocked
191 by adding 200 µl/well Blocking Buffer made of 1.5% (w/v) bovine albumine fraction V (BSA;
192 Merck, Darmstadt, Germany) in 10 mM PBS, pH 7.4, overnight at 4°C. The plate was
193 washed and incubated with standard dilutions in PBS (1, 10, 100 and 1000 ng/ml
194 osteocalcin) or undiluted sample extract (Table 1) for 2 h at room temperature (RT). The

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3 195 plate was washed and incubated with detection antibody (PAB OC1-9-Strep; 1:500 in PBS)
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5 196 for 2 h/RT, washed and incubated 1 h/RT with Biotinylated HRP (Invitrogen Corporation,
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7 197 Camarillo, CA; 1:10.000 in PBS). The plate was washed and the assay developed with
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9 198 chromogenic solution prepared as follows: 15 ml of citrate buffer (210 mM citric acid
10
11 199 monohydrate, 300 mM KOH, pH 3.95) and 13.6 mM freshly added H₂O₂ were mixed 20:1
12
13 200 (v/v) with 21.6 mM TMB (3,3',5,5'-Tetramethylbenzidine, BioChemica, AppliChem,
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15 201 Darmstadt, Germany) diluted in 2.5 ml acetone and 22.5 ml methanol (p.A.). After incubation
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17 202 for 20 min/RT in the dark the reaction was stopped by adding 1N H₂SO₄ to each well. The
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19 203 optical density was determined at 450 nm against air in a multimode plate reader (Mithras LB
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21 204 940, Berthold Tech. GMBH & Co. KG, Bad Wildbad, Germany). All samples and standards
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23 205 were measured in triplicates, statistical relevance between two data sets was calculated by
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25 206 two-side unpaired Student's t-test.
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28 208 *SDS-PAGE and Western blot*

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31 209 Discontinuous sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was
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33 210 performed with Tris-Glycin buffer according to Laemmli (1970) using a Mini Protean® Tetra
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35 211 System electrophoresis cell (Bio-Rad, Laboratories Inc., Germany). For the separation of low
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37 212 molecular weight proteins the separating gel contained 16.5% and the stacking gel 7.5%
38
39 213 acrylamide (Rotiphorese®Gel 30 [37.5:1]), Carl Roth GmbH & Co KG, Karlsruhe, Germany).
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41 214 Separated proteins in the gel were electro-transferred onto a nitrocellulose membrane
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43 215 (Amersham™ Hybond™-ECL, GE Healthcare) with a blotter unit (Bio-Rad, Laboratories Inc.)
44
45 216 at 4°C and 350mA/ 400V for 1 h using transfer buffer as described by the blotter unit's
46
47 217 manual. The blot was blocked in PBS/3 % BSA for 45 min at RT followed by an overnight
48
49 218 incubation at 4°C with PAB OC1-9-Strep diluted 1:500 in PBS/1.5 % BSA. The membrane
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51 219 was washed three times with TBST and PAB OC1-9-Strep binding onto the blot was
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53 220 detected by biotinylated HRP (1:5.000 in PBS containing 1.5 % BSA) after 1 h/RT incubation.
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55 221 After washing the blot was developed by adding 750 µl of a ready-mixed developing solution
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57 222 (Super Signal West Femto Maximum Sensitivity Substrate, Thermo Scientific) and chemo
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3 223 luminescence was measured with the Imaging System VersaDoc MP 4000 (Bio-Rad
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5 224 Laboratories, Munich, Germany).

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8
9 226 **Results**

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11 227 In order to develop a sandwich ELISA for the detection of processed bovine osteocalcin
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13 228 (OC), a rabbit polyclonal antibody was raised against the amino acid sequence 1-9 of pure
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15 229 bovine osteocalcin. The best performing pre-tested serum (PAB OC1-9) was mono-
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17 230 specifically purified. To confirm the reactivity of the isolated mono-specific IgG fraction, an
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19 231 immunoblot was carried out with an EDTA protein extract produced from native bovine bone.
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21 232 Solely one major low-molecular weight protein band (~5.8 kDa) was detectable
22
23 233 corresponding to the size of intact mature reference protein (Figure 2). No significant
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25 234 unspecific binding to other bone proteins occurred in bovine bone extract so that the antibody
26
27 235 PAB OC1-9 could be used to establish a sandwich ELISA pair with a monoclonal mouse
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29 236 capture antibody (OC4-30).

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33 238 The intra- and inter- assay variability and the sensitivity of the final ELISA set up was
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35 239 validated with standard dilutions of bovine osteocalcin in triplicates on three different days by
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37 240 the same operator (Figure 3). According to this validation experiment, the two-side assay is
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39 241 characterized by an average background signal ranging between $A_{450\text{ nm}}$ 0.170 – 0.260 for
40
41 242 the non template control (NTC; PBS instead of sample) and blank (PBS instead of capture
42
43 243 MAB). A significant signal above NTC and blank was obtained - with a probability of $p < 0.05$
44
45 244 in two-side Student's t-test - starting from an OC amount of 1 ng per well. Above this value
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47 245 the standard deviation is rising along with increasing signal strength, but the calculated inter
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49 246 assay coefficient of variation (2.8 – 23.3 %) (Figure 3) was considered satisfactory for all
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51 247 concentration levels and the assay accepted for the scope of investigation.

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56 249 EDTA extracts of native bone derived from most significant farmed animals as well as egg
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58 250 and milk protein and a compound plant feed used for BMBM spiking (Plant Feed-0 % BMBM)

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3 251 were measured to evaluate the specificity of the test. All EDTA-extracts were applied
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5 252 undiluted in ELISA, to ensure a maximum achievable response with highly processed
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7 253 materials or matrices of low MBM content. Protein yields typically varied between 0.6 – 2.8
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9 254 mg/ml for different bone and MBM extracts (Table 1). The EDTA extract with the highest
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11 255 protein amount was gained from egg powder with approximately 4 mg/ml.
12
13 256 The results of the specificity testing are summarized in Figure 4. The Limit of detection (LOD)
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15 257 was calculated as the mean absorption A_{450} value plus 3-fold standard deviation of 13
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17 258 different negative samples (measured in triplicate) which constitute typical feed compounds
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19 259 (plant feed, whole milk powder, hen`s egg) or potential contamination with other species than
20
21 260 cattle (fish meal, poultry meal, sheep MBM, sheep bone, pork MBM, pork bone, chicken
22
23 261 bone, goat bone, rabbit bone) as well as the NTC. The horse sample has been excluded
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25 262 from the calculation due to the 100 % amino acid identity with bovine osteocalcin and could
26
27 263 therefore not been considered as a negative sample. In all following experiments the LOD
28
29 264 indicates the baseline to discriminate true positive from negative results. The strongest
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31 265 ELISA signals ($A_{450} \text{ nm} > 3$) were detected for cattle and horse bone extracts. For these
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33 266 samples absolute protein amounts of 55 to 75 $\mu\text{g}/\text{reaction}$ were sufficient to elicit a strong
34
35 267 ELISA response with signals rapidly out-of-range if using further increased input amounts.
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37 268 Regarding bone extracts from other animals, a weaker but significant ELISA signal pointing
38
39 269 to an evident cross reaction was measured with 100 % native pork bone extract. In a western
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41 270 blot investigation with pure pig bone extract and PAB OC1-9 it was noticed, that a cross
42
43 271 reaction is directed against an unidentified protein of approximately 31 kDa (data not shown).
44
45 272 In addition, a weak cross reaction was also monitored for 100 % native sheep bone extract
46
47 273 (Figure 4). Bone extracts from chicken, goat or rabbit so as EDTA-extractable proteins from
48
49 274 hen`s egg, whole milk powder or compound plant feed all yielded negative results below
50
51 275 LOD-Baseline. EDTA-extracts derived from goose- or salmon bone, feather meal, as well as
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53 276 rye, maize and soybean were tested negative (data not shown).
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3 278 In the next step, the capacity of the immunoassay to detect OC in heat treated meat and
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5 279 bone meal extracts was investigated, to test the applicability of the ELISA on real life
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7 280 samples. Since no horse MBM was available, horse serum was used instead as an
8
9 281 exemplary matrix containing only trace amounts of osteocalcin. The total protein amount of
10
11 282 all employed undiluted MBM-extracts per ELISA well ranged in a comparable order of
12
13 283 magnitude between approx. 100 (poultry meal) – 280 µg (BMBM). The results are depicted in
14
15 284 Figure 5. All BMBM samples showed a pronounced immunological response, even if the
16
17 285 material was heated up to 145°C, whereas fish, poultry and sheep meal were tested
18
19 286 negative. Therefore the cross reaction with native sheep bone is judged negligible since it is
20
21 287 not interfering with the scope of the assay. A positive reaction was only detectable for
22
23 288 undiluted horse serum and – in contrast to sheep meal - for pork MBM extract. Though the
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25 289 immune response against porcine MBM was rather weak compared with BMBM (c.f. dog
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27 290 feed), the obtained pork MBM signal exceeded the LOD-baseline (Figure 5).
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31 292 The selectivity of the test was evaluated with extracts yielded from a standard plant feed
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33 293 spiked with 0.1 % (w/v) BMBM in comparison with the non-spiked matrix. In addition, since
34
35 294 no homogeneity approved material was available, dilutions of 0.1 % BMBM (dog feed) in fish
36
37 295 meal extracts were measured (Figure 6). The test discriminated 0.1 % (w/v; v/v) BMBM-
38
39 296 spiked plant feed or fish meal, respectively, from the non-spiked matrix. The quantitative
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41 297 content of osteocalcin in the plant feed spiked with 0.1 % (w/v) BMBM corresponds to 6.7 ng
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43 298 calculated in relation to the standard curve. Due to the observed cross reaction of the ELISA
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45 299 with pig meal, a separate selectivity experiment was performed. 0.1 %, 1 % and 10 % (v/v)
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47 300 BMBM (dog feed) extract in pig meal extract was measured on three days (Table 2). A
48
49 301 significant positive response was measurable for the 10 % (v/v) BMBM spiking level but not
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51 302 for the 1 % (v/v) spiked sample. The absorption measured with 100 % pork meal extract did
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53 303 not exceed the values corresponding to 10 ng bovine OC per well.
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58 305 **Discussion**
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3 306 A pre-requisite for the development of an immunological assay to detect fraudulent
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5 307 application to or unintended traces of MBM in feed is the thorough choice of a protein which
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7 308 exhibits exceptional resistance against mechanical forces, pressure and peak temperatures
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9 309 up to 140 – 150°C evoked by the rendering process (Pérez-Calvo et al. 2010; Garcia et al.
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11 310 2006). In this study the heat stable bone protein osteocalcin has proved to be a well suited
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13 311 MBM marker since a strong immunological signal was still detectable in BMBM even if
14
15 312 processed at 145°C. In addition, osteocalcin represents a genuine MBM- as well as animal
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17 313 marker protein since it is exclusively expressed in bone tissue or dentin in all vertebrates
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19 314 including bone fish (Hauschka et al. 1989).

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23 316 Though fairly stable if bound to the hydroxyapatite skeleton, osteocalcin under natural
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25 317 conditions undergoes rapid degradation in a turnover process in the blood plasma. Two
26
27 318 arginine-arginine cleavage sites are situated at the amino acid positions 19(Arg)-20(Arg) and
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29 319 43(Arg)-44(Arg) (Figure 1) resulting in two to three fragments (aa1-19, aa20-43, aa44-49) on
30
31 320 tryptic hydrolysis. The cleavage site near the C-terminal sequence is quickly hydrolysed
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33 321 cutting off the latter six amino acid residues whereas the resting aa1-43 fragment (N-MID
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35 322 fragment) is far more stable (Lee et al. 2000). Respecting the binding sites of the antibodies
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37 323 in our assay (aa1-9 and the epitope aa17) the results obtained with three different bovine
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39 324 MBM samples underline that a significant portion of at least the N-MID (aa1-43) is still
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41 325 immunologically accessible in MBM.

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45 327 To restrict the assay specificity to cattle, the synthetic N-terminal amino acid sequence aa1-9
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47 328 of bovine osteocalcin was used for the immunization of rabbits to exclude other most relevant
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49 329 farmed animals. The N-terminal sequences of osteocalcin in the genus *Bos*, *Sus*, *Ovis* and
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51 330 *Capra* differ only in one to three amino acids whereas this sequence is completely identical
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53 331 among *Bos* and *Equus* (Figure 1). The specificity testing confirmed that minor sequence
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55 332 differences were sufficient to limit the major polyclonal antibody reactivity to *Bos* with the
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57 333 predictable exception of *Equus*. However, an increasing tendency towards cross reaction is
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3 334 observed in accordance with the number of amino acid exchanges in relation to *Bos* OC aa1-
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5 335 9 ranking from *Capra* (3 exchanges): no cross reaction – *Ovies* (2 exchanges): very weak
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7 336 cross reaction – *Sus* (1 exchange): weak cross reaction - *Equus* (100 % sequence identity):
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9 337 strong cross reaction. The observed binding of the polyclonal rabbit IgG fraction to a 31 kDa-
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11 338 protein in western blot studies with PAB OC1-9 and porcine bone extract (data not shown)
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13 339 points rather to an unspecific side reaction to another protein than osteocalcin. To unravel
14
15 340 the nature of this cross reaction a mass spectroscopic investigation would be required which
16
17 341 was not subject of this study. However, the exchange of the voluminous aromatic amino acid
18
19 342 tryptophan at position aa5 in bovine OC against glycine in *Sus* could be targeted by the
20
21 343 production of a high affinity monoclonal antibody.
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23 344
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25 345 In the case of horse and cattle a sequence difference between both is given only at position
26
27 346 aa19 where lysine in bovine osteocalcin is exchanged by arginine in the genus *Equus*
28
29 347 (Figure 1). Therefore elimination of cross reaction in theory could be possible by an
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31 348 exchange of the capture antibody. However, an attempt to boost a polyclonal rabbit capture
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33 349 antibody against the synthetic mid-fragment spanning aa16 – 22 of bovine osteocalcin was
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35 350 not successful. This might be due to the lacking carboxylation of the glutamic acid residues
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37 351 at position 17, 21 and 24 of the synthetic peptide used for immunization, preventing
38
39 352 appropriate molecule folding. In addition the hydrophobic core of the molecule (Dowd et al.
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41 353 2003) most probably requires a conformational epitope rather than a sequence based
42
43 354 recognition site for antibody binding. The latter consideration is supported by a study using
44
45 355 monoclonal and polyclonal OC- directed antibodies which were tested with differently sized
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47 356 fragments of the molecule. The authors demonstrated a generally poor affinity of antibodies
48
49 357 against OC fragments comprising solely of aa11-29 or aa21-43 (Gundberg et al. 1998).
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51 358 Accordingly, the exclusion of horse- but also a reduction of pig cross reactivity could be
52
53 359 tackled by a compelling anti-protein production mode, e.g. as given by stringent phage
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55 360 display or monoclonal selection against native bovine osteocalcin fragments aa1-43, aa1-19,
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57 361 aa 4-19 or intact osteocalcin. The latter is planned for further investigations.
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5 363 Apart from osteocalcin, γ -carboxylated glutamic acids are occurring e.g. in proteins involved
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7 364 in the blood coagulation cascade like prothrombin, but could not be detected so far in
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9 365 hemolymph of annelids, crustaceous organisms, insects, soybean leafs, *E. coli* extract or
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11 366 bovine milk (Zytkovicz and Nelsestuen 1976). Accordingly, the ELISA developed in this study
12
13 367 verified a remarkable selectivity for osteocalcin in plant background. A protein extract
14
15 368 prepared from a classical feed mix used for the preparation of 0.1 % BMBM spiked feed
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17 369 samples, yielded a background signal comparable with blank and NTC. Similar results were
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19 370 obtained with 100 % soybean-, wheat- or corn protein extracts and a further plant based milk
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21 371 replacer containing 12 % citrus and 5 % cocoa shell (data not shown). The presence of beet
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23 372 or citrus pulp obviously does not pose a problem in contrast to test interference observed for
24
25 373 these ingredients in a previous validation study on commercial test kits (Boix et al. 2004).
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27 374 The selectivity of the assay was also excellent in fish meal background which is explainable
28
29 375 by the pronounced sequence differences between osteocalcin from bone fish compared with
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31 376 terrestrial animals (Figure 1). In contrast, the observed weak but significant cross reactivity
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33 377 with pure pig bone or pig meal decreased the selectivity of the assay. However, only one
34
35 378 sample of pure pork meal was available for testing so that further pork MBM materials as well
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37 379 as defined mixtures have to be investigated to verify this result. Due to the nature of the
38
39 380 material a cross-contamination with bovine material could not be excluded. The identification
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41 381 of MBM traces derived from other species – e.g. BMBM in pork or fish meal – seems to be a
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43 382 most challenging issue associated with MBM targeted immunological methods which was
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45 383 also observed for commercial cooked meat kits. In studies on the performance on two
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47 384 broadly distributed dip stick tests increasingly false positive results were monitored in the
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49 385 presence of 5 % pork meal or animal fat or with fish meal at levels as low as 1.5 % (Fumière
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51 386 et al. 2009; Boix et al. 2004).
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55 388 The percentage of bone fragments in two defined BMBM samples which we used in our
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57 389 experiments was about 54 and 80 % (w/w), which is the common range reported for
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3 390 materials from rendering plants (Liu et al. 2010). The good results yielded with EDTA as a
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5 391 bone solvent is in line with recently published results on an improved extraction method for
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7 392 the detection of ruminant material. Due to an EDTA-based extraction mode the performance
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9 393 of a commercial dip stick test could be improved from 2 % down to a detection level of 0.1 %
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11 394 BMBM in feed (Karanam 2011). In contrast to our approach the method includes a
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13 395 concentration step of the bone fraction by sedimentation with 100 ml tetra chloroethylene
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15 396 (TCE) as used in sample preparation for microscopy (Regulation (EC) No 152/2009). An
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17 397 additional density based concentration of bone fragments is not necessary for the ELISA
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19 398 method described here avoiding an agent supposed to be carcinogenic (IPCS INCHEM;
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21 399 <http://www.inchem.org/>). However, an over night bone digestion step which does not allow
22
23 400 rapid sample analysis within a few hours, was also applied in our study. To speed up the
24
25 401 extraction method used here the procedure could possibly be optimized by hot and/or acid
26
27 402 extraction or augmentation of the sample amount or further homogenization of coarse
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29 403 fractions.

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33 405 The bone content might play an essential role regarding the sensitivity of the method. The
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35 406 question if an osteocalcin based method would also perform with samples containing < 54 %
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37 407 (w/w) bone or even with 100 % muscle meal remains to be clarified with appropriate
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39 408 materials which were not available in this study. With respect to the expectable great
40
41 409 variance of MBM from different rendering plants it would be anyhow advisable not only to rely
42
43 410 on one single target molecule but to apply a panel of well defined marker-proteins to capture
44
45 411 the great diversity of animal by-products produced by the rendering industry.

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47 412
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49 413 Although the sensitivity of our assay 1 ng bovine OC is about one order of magnitude below
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51 414 commercial osteocalcin ELISA validated for pharmaceutical applications, the sensitivity with
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53 415 MBM looks encouraging compared with commercial ELISA test kits developed for the scope
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55 416 of species detection in processed meat. For these, sensitivities of 0.1, 0.5 or ≥ 2 % MBM in
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57 417 feed or even a complete failure to detect legally treated MBM have been reported from
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3 418 diverse validation studies (Boix et al. 2004; Karanam 2011; Meyers et al. 2007; von Holst et
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5 419 al. 2006; Rao and Hsieh 2007; Love and Carey-Smith 2004; Gizzi and von Holst 2004). The
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7 420 sensitivity of the ELISA procedure used in our study primarily might have been decreased
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9 421 during the mono-specific purification step retaining most potent binding IgG from the
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11 422 polyclonal sera. The linkage to the voluminous streptavidine molecule had no negative
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13 423 impact on the sensitivity compared with horseradish peroxidase labelling (data not shown)
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15 424 but an enhancing effect due to multiple biotinylation sites.
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18 19 426 **Conclusions**

20
21 427 In conclusion our results demonstrate that osteocalcin is a promising tissue specific target
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23 428 protein for the sensitive immunological detection of meat and bone meal in compound feed
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25 429 or fish meal with an excellent selectivity for BMBM in these matrices. Furthermore
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27 430 environmental bone fragment contaminants in sugar beet epidermis, e.g. derived from rabbit,
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29 431 rat or mouse can be easily discriminated because of marked sequence divergences to e.g.
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31 432 bovine osteocalcin. The here described antibody-based osteocalcin approach might be the
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33 433 basis for a validated detection tool for official law enforcement of the feed ban. Moreover the
34
35 434 general approach using epitop-specific antibodies is target-directed and might be an
36
37 435 improvement in terms of the acceptance as national or international standard in the future.
38
39 436 Moreover quantification is possible based on a standard curve established by commercially
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41 437 available pure bovine osteocalcin.
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44 45 439 **References**

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3 **Figure 1.** Comparison of osteocalcin sequences published for farmed animals, pets, rodents,
4 amphibian and bone fish species. Conserved carboxylated glutamic acid residues at position
5 17, 21 and 24 are asterisked. Black arrows point to tryptic cleavage sites. Amino acid
6 exchanges between *Bos* and other farmed animals are highlighted in bold types. The
7 position of the sequence targeted by the detection antibody PAB OC1-9 and the N-MID-
8 fragment (1-43) are marked by black lines. The capture antibody OC4-30 (Takara) used in
9 the sandwich assay in this study binds to the epitope around position 17.

10
11 **Figure 2.** Specificity of PAB OC1-9 (polyclonal anti-osteocalcin IgG-fraction) towards native
12 bovine bone protein in western blot analysis. M, Lane 2: Low-Range Molecular Weight
13 Marker (3.5-40 kDa), Amersham, GE Healthcare; Lane 1: NTC (water instead protein); Lane
14 3: OC=Positive Control (bovine osteocalcin, 1.3 µg/slot); Lane 4: BB=bovine bone EDTA
15 extract (7.2 µg/slot)

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17 **Figure 3.** Osteocalcin specific sandwich ELISA with MAB OC4-30 (Takara) as capture and
18 PAB OC1-9 as detection antibody. Sensitivity with purified bovine osteocalcin. Error bars
19 indicate a one-fold standard deviation. Measurements were done in three replicates on three
20 different days. Blank=PBS instead of capture; NTC= PBS instead of sample; *significant
21 difference ($p < 0.05$) against NTC and Blank.

22
23 **Figure 4.** Osteocalcin specific sandwich ELISA with MAB OC4-30 (Takara) as capture and
24 PAB OC1-9 as detection antibody. Specificity with EDTA-extracts from native bone and feed
25 components. Error bars indicate a one-fold standard deviation. Undiluted EDTA extracts
26 were measured in triplicate. Horizontal line = LOD = Mean + 3x SD from 13 negative
27 samples as indicated in the results section.

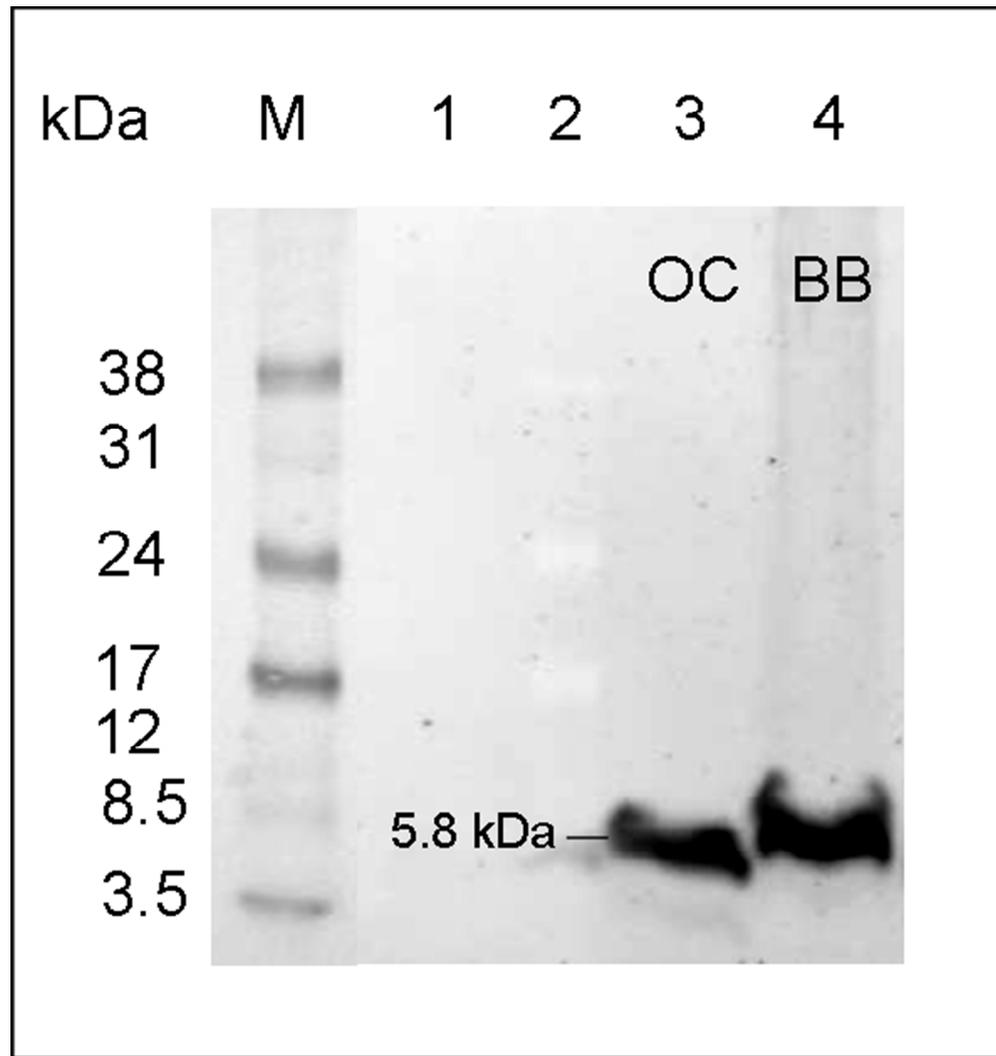
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29 **Figure 5.** Osteocalcin specific sandwich ELISA with MAB OC4-30 (Takara) as capture and
30 PAB OC1-9 as detection antibody. Method performance with meat and bone meal extracts.
31 Error bars indicate a one-fold standard deviation. Undiluted EDTA extracts were measured in
32 triplicate. Horizontal line = LOD = Mean + 3x SD from 13 negative samples as indicated in
33 the results section.

34 **Figure 6.** Osteocalcin specific sandwich ELISA with MAB OC4-30 (Takara) as capture and
35 PAB OC1-9 as detection antibody. Selectivity in mixtures containing 0.1 % bovine MBM.
36 Error bars indicate a one-fold standard deviation. Undiluted EDTA extracts were measured in
37 triplicate. Horizontal line = LOD = Mean + 3x SD from 13 negative samples as indicated in
38 the results section.
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Species:	N-MID-fragment		NCBI Accession:
	1	43	
<i>Bos taurus</i>	YLDH--WLGAPAPYPDPLEPKREVCELNPDCELDADHIGFQEA [*] YRRFYGPV--		ABU88822.1
<i>Ovis aries</i>	YLDP-- GLGAPAPYPDPLEPR REVCELNPDCELDADHIGFQEA [*] YRRFYGPV--		ABD83814.1
<i>Capra hircus</i>	YLDP-- GLGAPAPYPDPLEPK REVCELNPDCELDADHIGFQEA [*] YRRFY GLA --		1005180A
<i>Sus scrofa</i>	YLDH-- GLGAPAPYPDPLEPR REVCELNPDCELDADHIGFQEA [*] YRRFY GLA --		Q8HY9.2
<i>Equus caballus</i>	YLDH--WLGAPAPYPDPLEPKREVCELNPDCELDADHIGFQEA [*] YRRFYGPV--		P83005.1
<i>Gallus gallus</i>	HYAQDSGVAGA--PPNPLEAQREVCELSPDCELDADQIGFQEA [*] YRRFYGPV--		AAA78809.1
<i>Dromaius novaehollandiae</i>	--SFAVGSSYGA--APDPLEAQREVCELNPDCELDADHIGFQEA [*] YRRFYGPV--		P15504.1
<i>Rattus norvegicus</i>	YLMN--GLGAPAPYPDPLEPHREVCELNPNCELDADHIGFQDAYKRIYGTTV--		AAA53280.1
<i>Mus musculus</i>	-----YLGASVPSDPDPLEPTREQCELNPADELSDQVGLKTAYKRIYGITI--		AAA39856.1
<i>Canis familiaris</i>	YLDS--GLGAPVPYPDPLEPKREVCELNPNCELDADHIGFQEA [*] YRRFYGPV--		AAB27444.1
<i>Felis sylv. catus</i>	YLAP--GLGAPAPYPDPLEPKREICELNPDCELDADHIGFQDAYRRFYGTV--		P02821.1
<i>Oryctolagus cuniculus</i>	QLIN--GQGAPAPYPDPLEPKREVCELNPDCELDADQVGLQDAYQRFYGPV--		P39056.1
<i>Xenopus laevis</i>	--SYGNVVGQGAAVGSPLESQREVCELNPDCELDADHIGFQEA [*] YRRFYGPV--		AAB36024.1
<i>Salmo salar</i>	AGTALADLSL-----TQLESREVCELN [*] YWCENMMDTAGIIAAYTEFYGPPIY		NP_001130023.1
<i>Oncorhynchus mykiss</i>	ASTALADLYL-----TQLESREACELN [*] YWCENMMDTAGIIAAYTEFYGPPIY		AAPO4487.1
<i>Scophthalmus maximus</i>	---AAGELSL-----TQLESREVCELN [*] LACEEMMDTGGIIAAYNAFYGPPIY		AAV80230.1
<i>Diplodus puntazzo</i>	---AAGQLSL-----TQLESREVCELN [*] LACEHMDTEGGIIAAYTAYYGPPIY		AAAY17284.1
<i>Sparus aurata</i>	---AAGQLSL-----TQLESREVCELN [*] LACEHMDTEGGIIAAYTAYYGPPIY		AAK66568.1
<i>Pagrus auriga</i>	---AAGQLSL-----TQLESREVCEAN [*] LACEDMMDTEGGIIAAYTAYYGPPIY		AAV80232.1
<i>Halobatrachus didactylus</i>	---AAELSL-----VQLESPREVCE [*] TNVACDEMMDTGGIIIVAYTAYYGPPIY		AAQ13575.1
<i>Argyrosomus regulus</i>	---AAKELTL-----AQTESREVCE [*] TNMACDEMADAQGGIVAAAYQAFYGPPIY		AAO48725.1
<i>Macropus eugenii</i>	--YLYQTLGFAPYPDPQENKREVCELNPDCELDADHIGFQEA [*] YRRFYGTA--		POC226.1
<i>Lepomis macrochirus</i>	---AAGELTL-----TQLESREVCEAN [*] LACEDMMDAQGGIIAAYTAYYGPPIY		AAB22263.1

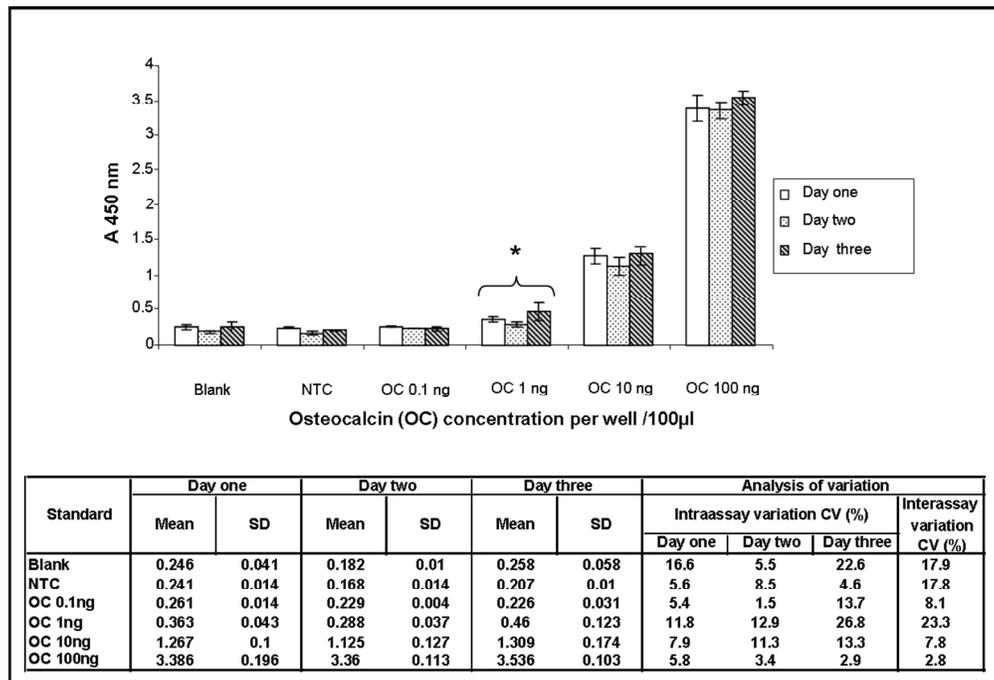
Comparison of osteocalcin sequences published for farmed animals, pets, rodents, amphibian and bone fish species. Conserved carboxylated glutamic acid residues at position 17, 21 and 24 are asterisked. Black arrows point to tryptic cleavage sites. Amino acid exchanges between *Bos* and other farmed animals are highlighted in bold types. The position of the sequence targeted by the detection antibody PAB OC1-9 and the N-MID-fragment (1-43) are marked by black lines. The capture antibody OC4-30 (Takara) used in the sandwich assay in this study binds to the epitope around position 17.

248x164mm (150 x 150 DPI)

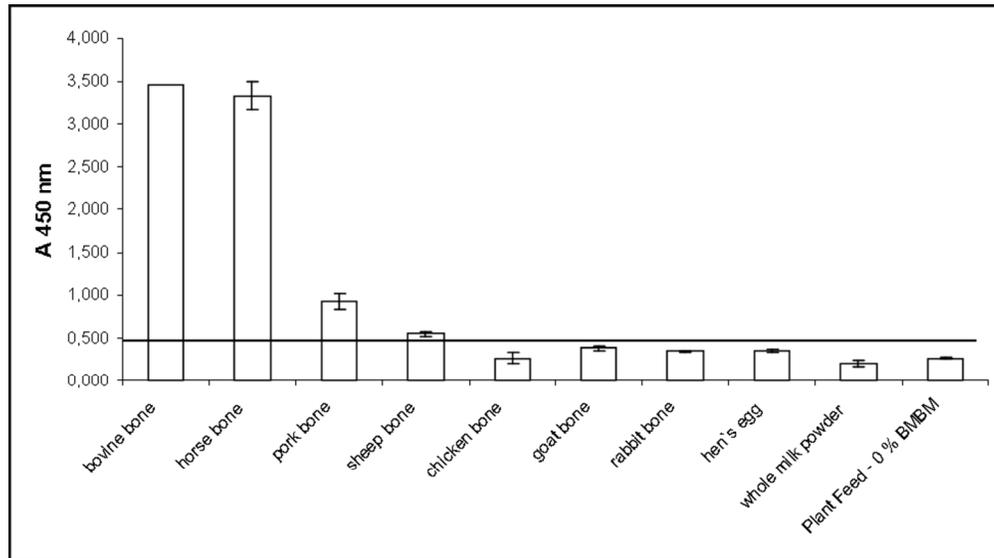


42 Specificity of PAB OC1-9 (polyclonal anti-osteocalcin IgG-fraction) towards native bovine bone protein in
43 western blot analysis. M, Lane 2: Low-Range Molecular Weight Marker (3.5-40 kDa), Amersham, GE
44 Healthcare; Lane 1: NTC (water instead protein); Lane 3: OC=Positive Control (bovine osteocalcin, 1.3
45 $\mu\text{g}/\text{slot}$); Lane 4: BB=bovine bone EDTA extract (7.2 $\mu\text{g}/\text{slot}$)
46 100x106mm (150 x 150 DPI)

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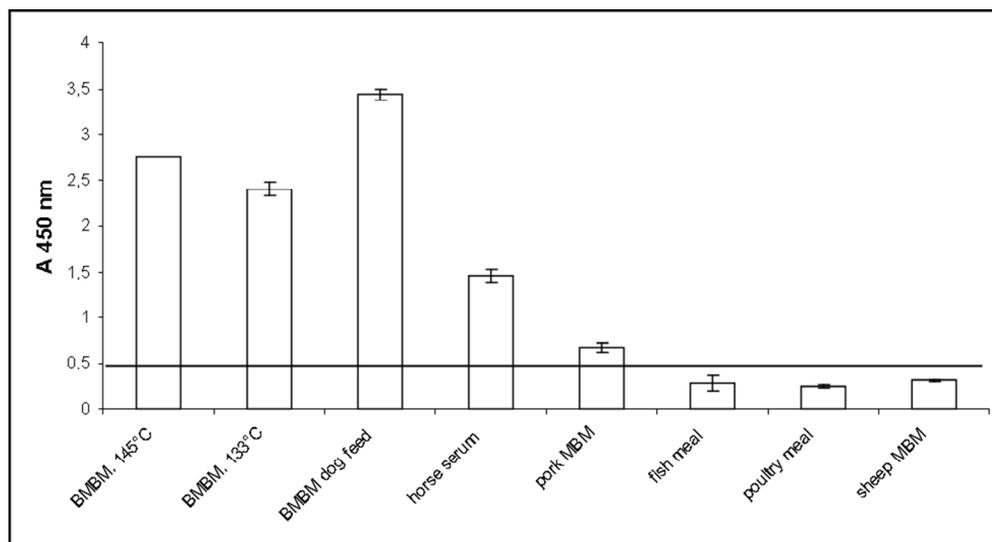


Osteocalcin specific sandwich ELISA with MAB OC4-30 (Takara) as capture and PAB OC1-9 as detection antibody. Sensitivity with purified bovine osteocalcin. Error bars indicate a one-fold standard deviation. Measurements were done in three replicates on three different days. Blank=PBS instead of capture; NTC= PBS instead of sample; *significant difference ($p < 0.05$) against NTC and Blank.
210x143mm (150 x 150 DPI)



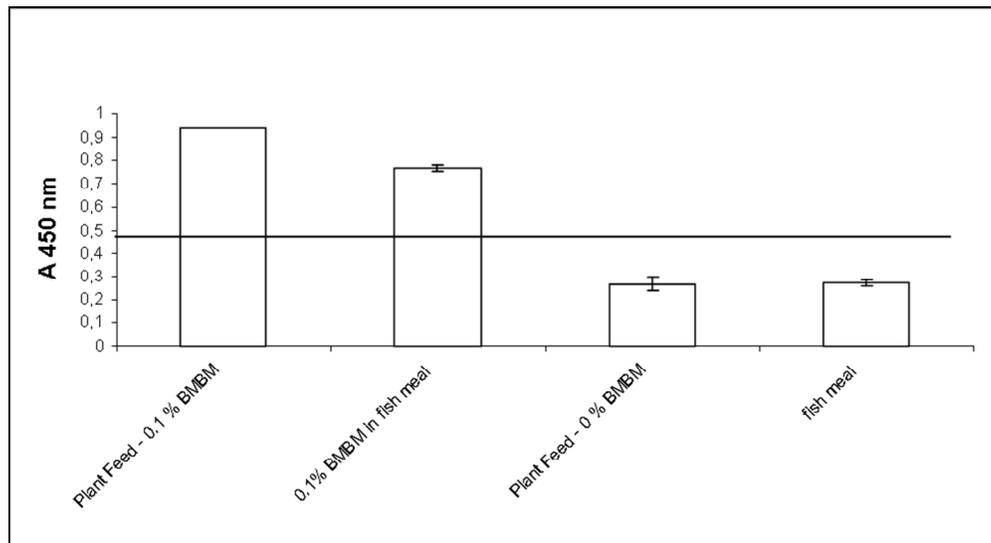
Osteocalcin specific sandwich ELISA with MAB OC4-30 (Takara) as capture and PAB OC1-9 as detection antibody. Specificity with EDTA-extracts from native bone and feed components. Error bars indicate a one-fold standard deviation. Undiluted EDTA extracts were measured in triplicate. Horizontal line = LOD = Mean + 3x SD from 13 negative samples as indicated in the results section.

165x92mm (150 x 150 DPI)



Osteocalcin specific sandwich ELISA with MAB OC4-30 (Takara) as capture and PAB OC1-9 as detection antibody. Method performance with meat and bone meal extracts. Error bars indicate a one-fold standard deviation. Undiluted EDTA extracts were measured in triplicate. Horizontal line = LOD = Mean + 3x SD from 13 negative samples as indicated in the results section.

166x90mm (150 x 150 DPI)



Osteocalcin specific sandwich ELISA with MAB OC4-30 (Takara) as capture and PAB OC1-9 as detection antibody. Selectivity in mixtures containing 0.1 % bovine MBM. Error bars indicate a one-fold standard deviation. Undiluted EDTA extracts were measured in triplicate. Horizontal line = LOD = Mean + 3x SD from 13 negative samples as indicated in the results section.

166x90mm (150 x 150 DPI)

Table 1. Protein concentration of EDTA extracts used in ELISA

Sample	Protein concentration of the undiluted extract used per well [mg]
BMBM. 145°C	0.278
BMBM. 133°C	0.283
BMBM dog feed	0.124
Plant Feed 0.1% (w/w) BMBM	0.094
Plant Feed 0% BMBM	0.068
fish meal	0.193
pork MBM	0.124
sheep MBM	0.182
poultry meal	0.098
bovine bone	0.055
sheep bone	0.163
goat bone	0.267
pork bone	0.242
chicken bone	0.182
rabbit bone	0.117
horse bone	0.075
horse serum	0.878
hen`s egg	0.406
whole milk powder	0.231

Table 2. Selectivity in osteocalcin specific sandwich ELISA. using MAB OC4-30 (Takara) as capture and PAB OC1-9 as detection antibody. for hidden BMBM in pork meal in three independent measurements

Extract	Day 1	Day 2	Day 3	Mean	SD
BMBM dog feed	3.469	3.352	3.501	3.441	0.078
Pork MBM	0.677	0.753	0.586	0.672	0.084
10% BMBM in pork MBM	0.887	1.116	1.232	1.078	0.176
1% BMBM in pork MBM	0.525	0.561	0.595	0.560	0.035
0.1% BMBM in pork MBM	0.476	0.524	0.514	0.505	0.025

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