Analytical Methods

Selected reaction monitoring method to determine the species origin of blood-based binding agents in meats: A collaborative study

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Abstract

Binding products or food ‘glues’ are used throughout the food industry to increase the meat use rate or to augment economic efficiency. Some of these binders contain thrombin from bovine and porcine blood. The European parliament has recently banned thrombin-based additives and labelling legislation governs their use in the US. A mass spectrometry screening method is available to detect the addition of thrombin agents to foods as there is a need to protect consumers and to avoid misleading trade practices. We report the details of an inter-laboratory trial to determine the transferability of this method to operators in various food testing laboratories, each using a different triple quadrupole mass spectrometer design. The trial was successful with the species origin of the binding agent contained in each of the 43 test materials being correctly reported by the participants. This is consistent with a false positive and false negative rate of 0%. This is the first collaborative study, as far as we are aware, which involves a liquid chromatography mass spectrometry (LC–MS/MS) application to approach a food authenticity issue.

1. Introduction

There is an increasing demand in the food industry for accurate portion control in meat and fish products. In an attempt to meet demand while maximising the use of lower value carcase meats, re-structuring technologies have been developed that utilise binders to incorporate lower value trimmings into a standard sized consumer product. Certain binders or ‘food glues’ are prepared from extracts of the blood plasma of cow or pig carasses and are supplied as fibrinogen and thrombin suspensions which, once combined, clot around the meat to bind it together in much the same way as blood coagulates in vivo. There was concern that there was an opportunity for unscrupulous producers to use this type of binding agent to fraudulently increase the declared meat content of products. This particular food binding process also raises ethical and religious issues since there is potential for derivatives from pig or cow to be added as an undeclared processing aid in the manufacture of other meat and fish products. The European Parliament has recently banned thrombin-based food additives from use (URL http://www.eurofoodlaw.com/food-safety-and-standards/meps-block-meat-glue-approval–1.htm?origin=internal Search. Accessed 01.12.12). Although these binders are permitted for use in the USA, US legislation requires the retail food label to contain the words ‘formed’ or ‘bonded’, the species of origin and the tissue, for example: ‘formed with beef fibrin’ (URL http://www.fsis.usda.gov/OPPDE/larc/Policies/Labeling_Policy_Book_082005.pdf. Accessed 16.01.13). A method to screen for the presence of this binding agent is therefore required to enforce legislation in the UK, the rest of the European Union and on an international basis to avoid misleading trade practices.

A qualitative selected reaction monitoring (SRM) method was recently developed to determine the species origin of blood-based binding agents in a range of food matrices at the request of the UK Food Standards Agency (Grundy et al., 2007, 2008). This SRM method is based on the fact that, during the blood clotting process, two peptides known as fibrinopeptides A and B are released and the amino acid sequences, masses, and ion fragmentation patterns of these peptides are unique for many species (Benson, Karsh-Mizrachi, Lipman, Ostell, & Wheeler, 1981). Fibrinogen, the main protein of the blood-based binding agent, is composed of three pairs of non-identical peptide chains, the
overall structure being (Aα, Bβ, and γ2). Fibrinogen is cleaved by the blood protease thrombin, which first removes a pair of peptides from the N-terminus of the α chains (fibrinopeptides A), then a second pair of peptides from the N-terminus of the β-chains (fibrinopeptides B) (Blomback, Hessel, Hogg, & Therkildsen, 1978).

Details of the peptide sequences of bovine and porcine fibrinopeptides screened for in this method are shown in Table 1. Bovine fibrinopeptide B has a pyroglycamin residue at the N terminus. The screening method is capable of differentiation between interstitial blood in the meat, which intrinsically occurs at a relatively low level, and the addition of blood-based binders, for which the manufacturers recommend addition at a level of 10–30%. Concerning the sensitivity of the method, from previous unpublished work, it is known that the species origin of blood-based binding agent can be determined in foods containing as little as 5% (v/w) of the agent. Also, the stability of the fibrinopeptides in the binding agent when added to foods was determined at least 10 days in meats stored under refrigerated conditions and at least 200 days in meats stored in a domestic freezer (–20 °C nominal).

Now that a method is available, in order to gain further information regarding the false negative and false positive rates and also to determine the transferability of the technique to various instruments in a range of laboratories, a collaborative study was organised. Participants from food testing laboratories across Europe and in the USA took part. Although ring trials have taken place using LC–MS/MS to address food safety issues (MacDonald, Chan, Brereton, Damant, & Wood, 2005; Wenzl et al., 2006), this is the first inter-laboratory study, as far as we are aware, which involves LC–MS/MS to approach a food authenticity issue. Other food authenticity collaborative studies have taken place using other techniques, with examples in isotope ratio mass spectrometry and protein gel electrophoresis (Mackie, Jamin, Martin, & Martin, 2004), Real Time PCR (Hird, Powell, Johnston, & Oehlschlager, 2003) and protein gel electrophoresis (Mackie et al., 2000) to name but a few.

2. Materials and methods

In the co-ordinating institute where test materials were prepared and pre-screened, all materials were purchased from Sigma–Aldrich Company Limited unless otherwise stated.

2.1. Synthetic peptide standards

Synthetic bovine and porcine fibrinopeptides A and B were prepared by Severn Biotech Ltd., UK as detailed in Table 1, 85% purity.

2.2. Standard operating procedure

A standard operating procedure (SOP) was dispatched to participating laboratories which detailed the extraction procedure, LC–MS/MS conditions and quality assurance information as detailed below. Since the exact LC–MS/MS conditions vary according to the instrument used, during an initial preparatory and training round, each participant optimised the LC–MS/MS method on their instrument using bovine and porcine fibrinopeptide A and B standards prior to implementing the extraction and screening methods on supplied food samples. Suggested transitions were detailed in the SOP for each fibrinopeptide but participants established the optimal transitions for their particular instrument as detailed in Table 2. In this way, participants could also tune their instruments to account for chemical effects such as deamidation. An applicable LC–MS/MS method is detailed below. The LC–MS/MS instruments used by participants in the trial included the 4000 QTRAP (AB Sciex), TSQ Quantum Ultra (Thermo Scientific Inc.), Ultima Quattro Pt (Waters Corp., originally Micromass Ltd.) and XEVO TQ-S (Waters Corp.).

2.2.1. Extraction of fibrinopeptides from test materials

Test materials (2 g aliquot) were homogenised with 6.2 mL of chilled (4 °C) trichloroacetic acid (6.2%, v/w) and precipitated on ice. Following centrifugation at 4000g the supernatant was washed twice with an equal volume of diethyl ether (Fisher Scientific Ltd.) and then once with butanol (Fisher Scientific Ltd.) followed by hexane (Fisher Scientific Ltd.). The peptide solution was then lyophilised and reconstituted in 1 mL of phosphate buffered saline (0.009 M phosphate buffer, 0.0024 M potassium chloride, 0.0012 M sodium chloride, 0.1 M potassium dihydro-orthophosphate, pH 7.2). The suspension was mixed vigorously on a vortex shaker at room temperature for 1 h. Undissolved solids were removed by centrifugation at 15000g for 2 min. The sample was applied to an Oasis® 30 mg HLB (hydrophilic–lipophilic-balanced) cartridge (Waters Corp.) as per the manufacturer’s instructions to extract interfering acidic, basic, and neutral compounds. Peptides bound to the cartridge were washed with 10% methanol in water prior to elution from the cartridge in 40% methanol, 2% ammonium hydroxide in water. This peptide sample was then transferred to an Oasis® mixed-mode polymeric sorbent MAX cartridge (Waters Corp.) to extract acidic compounds with anion-exchange groups. The loaded cartridge was washed with 40% methanol, 0.2% acetic acid pH 5.2 in water prior to peptide elution in 40% methanol, 0.2% formic acid, pH 3.2 in water. The eluant was dried in a centrifugal evaporator prior to reconstitution and analysis.

2.2.2. LC–MS/MS method

A suitable method for the 6490 triple quadrupole LC–MS/MS (Agilent Technologies) is as follows. Reversed phase separation occurred on an Agilent Technologies Poroshell 120 SB C18 2.7 µm (2.1 x 100 mm) column with filter with acetonitrile/aqueous-formic acid, pH 2.2 gradient. Starting conditions were 4% acetonitrile/96% aqueous formic acid, held for 4 min and rising to 31% acetonitrile/formic acid over 7 min and held at this concentration for a further 7 min prior to a column cleaning step with 95% acetonitrile/formic acid. Acetonitrile was produced by Mallinckrodt Baker Inc. and formic acid was produced by Biosolve Chemicals. Type 1+ grade water (resistivity of 18.2 MΩ-cm) was used. The LC flow rate was 0.3 mL/min with a 15 µL injection. The mass spectrometer used the electrospray source in positive ionisation mode with a capillary voltage of 3.5 kV, sheath gas temperature of 250 °C, sheath gas flow at 11 L/h, drying gas temperature of 300 °C and drying gas flow at 5 L/min. Cone voltages and collision energies were set for individual selected reaction monitoring transitions. Bovine fibrinopeptide A was determined by monitoring three transitions for each peptide.
transitions, 946.6 > 695.0, 946.6 > 445.0 and 946.6 > 357 where the precursor ion corresponded to the diprotonated molecule. Three transitions were monitored for fibrinopeptide B, 783.3 > 685.1, 783.3 > 314.1 and 783.3 > 296.2, where the precursor ion corresponded to the triprotonated molecule. Porcine fibrinopeptide A was determined by monitoring three transitions, 588.2 > 574.4, 588.2 > 201.1 and 588.2 > 157.0 where the precursor ion corresponded to the triprotonated molecule. Fibrinopeptide B was determined by monitoring five transitions, 734.0 > 246.0 and 734.0 > 157.0 where the precursor ion corresponded to the triprotonated molecule and 551.0 > 251.3, 551.0 > 157 and 551.0 > 136.0 where the precursor ion corresponded to the protonation state of four.

2.3. Test materials

Bovine and porcine blood-based binding agents were sourced from Fibrisol Service Ltd, supplied in the form of separate fibrinogen and thrombin suspensions.

Training round – lamb meat was supplied and removed from the bone by a local butcher. The meat was homogenised to a pulp and aliquots were taken for pre-screening to check that matrix interferences did not interfere with analysis. A portion of the meat was spiked in-house with porcine-derived binding agent (10% v/w). This homogenised flesh material was separated into aliquots containing 10 g of test material which were assigned unique (non-descriptive) identifier numbers. Two aliquots were selected at random and analysed to check that the species origin of the fibrinopeptides could be correctly assigned on the test materials by this qualitative method. Forty three ‘blind’ test materials in total were then distributed at random (assigned using random numbers tables) among the five participants for analysis with each laboratory receiving between seven and nine test materials.

2.4. Acceptance criteria

The LC–MS/MS analysis sequence was provided in the SOP, including solvent blanks, calibration series, and samples. The first injection of any run sequence was a solvent blank to show the system was ‘clean’. A standard curve of the LC–MS/MS transition peak areas of three concentrations (0.1, 1.0 and 10.0 μg/mL) of working solution reference material was included at the beginning and after every ten injections in a batch and it was required that $R^2 > 0.9$. This was followed by an injection of a solvent blank to show there was no carryover of analyte in the injection system. The standard curve and a solvent blank were also injected at the end of every batch of test materials. The lowest point on the standard curve, and thus the minimum peak intensity at which a transition was reported as detected, was equivalent to that of 0.1 μg/mL of fibrinopeptide. The relative intensities of the detected transitions, expressed as a percentage of the intensity of the most intense ion ratio, were required to correspond to that of the mean of the calibration standards, measured under the same conditions, within the following tolerances: ion ratio >0.50 = maximum permitted tolerance of ±20%, 0.20–0.50 = ±25%, 0.10–0.20 = ±30% and <0.10 = ±50%.

In addition to the above, in order to report the detection of a blood-based binding agent, the observation of one transition was required and it was necessary that it eluted at the expected retention time of the reference standard (±5%). The peak area was

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Table 2
Details of all of the transitions determined by the participating laboratories in order to screen for the bovine and porcine fibrinopeptides A and B in the fibrinopeptide standards.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Precursor ion, m/z (±1.0)</th>
<th>Product ion, m/z (±1.0)</th>
<th>Instruments associated with transitiona</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine fibrinopeptide A, [M + 2H]2+</td>
<td>946.6</td>
<td>695.5, 455.0, 357.3, 227.1, 120.0</td>
<td>1, 2, 3, 4, 5</td>
</tr>
<tr>
<td>Bovine fibrinopeptide B (pE), [M + 3H]3+</td>
<td>783.3</td>
<td>797.5, 685.1, 697.4, 314.1, 296.2, 171.0</td>
<td>1, 2, 3, 4, 5</td>
</tr>
<tr>
<td>Porcine fibrinopeptide A, [M + 3H]3+</td>
<td>588.2</td>
<td>574.4, 445.3, 201.1, 157.2</td>
<td>1, 2, 3, 5</td>
</tr>
<tr>
<td>Porcine fibrinopeptide A, [M + 2H]2+</td>
<td>881.4</td>
<td>201.1, 1091.5</td>
<td>2, 3</td>
</tr>
<tr>
<td>Porcine fibrinopeptide B, [M + 4H]4+</td>
<td>551.0</td>
<td>251.3, 157.0, 136.2</td>
<td>1, 2, 3, 4, 5</td>
</tr>
<tr>
<td>Porcine fibrinopeptide B, [M + 3H]3+</td>
<td>734.0</td>
<td>1008.5, 246.2, 185.1, 157.2, 136.2</td>
<td>1, 2, 3, 4, 5</td>
</tr>
</tbody>
</table>

* Details of the instruments with which each transition was determined: Instrument 1. 4000 Q Trap LC/MS/MS (AB Sciex), 2. 6490 triple quadrupole MS (Agilent Technologies), 3. TSQ Quantum Ultra Triple Stage Quadrupole MS (Thermo Scientific), 4. Quattro Ultima Pt MS (Waters Micromass), 5. Xevo TQ-S (Waters Corporation).
required to be greater than that of the corresponding fibrinopeptide standard in the 0.1 \( \mu \text{g/mL} \) injection. Confirmation of the presence of the specific binding agent was dependent on the detection of at least two fibrinopeptide transitions from the same species, either as two transitions from the same fibrinopeptide A or as one transition from the fibrinopeptide A and one from the fibrinopeptide B.

2.5. Management of collaborative study

Initially eight laboratories were enlisted to participate in the study. However, due to other priorities, only five laboratories took part in the inter-laboratory trial. The trial involved an initial training round in which each participant was sent two ‘blind’ test materials to analyse in duplicate. Participants were required to analyse the samples and submit the determined species of origin on a results proforma. Participants also submitted their MS data so that it could be verified that all acceptance criteria had been met. Participants were required to successfully pass this initial round before moving onto the final inter-laboratory trial round. Again, the determined species of origin for each test material was submitted on a results proforma along with all of the MS data for to verify that the data of submitted results adhered to the acceptance criteria.

3. Results and discussion

3.1. Initial training round

Firstly, participants optimised the LC–MS/MS method using fibrinopeptide standards. Since each participant used a different triple quadrupole mass spectrometer, various transitions were observed and screened for in different laboratories for the four fibrinopeptides. Examples of the ESI mass spectra of the product ions for the fibrinopeptide standards for one of the participants are shown in Fig. 1. A full list of the transitions observed for the fibrinopeptide standards during the inter-laboratory trial are detailed in Table 2.

All five participants correctly reported the species provenance of the two test materials (one bovine and one porcine test material) in the initial training round and met the acceptance criteria. Participants observed only one or two transitions relating to bovine blood binding agent (Fig. 2) but a higher number (four to six transitions) for the porcine agent in the test materials. In all cases, the bovine transitions observed related to fibrinopeptide A rather than fibrinopeptide B. This apparent prevalence of bovine fibrinopeptide A in foods containing blood-based binding agent has been reported previously (Grundy et al., 2007). A possible explanation is that, as described above, during the blood clotting process, fibrinopeptide A is the first peptide to cleave from the fibrinogen protein (Blombäck et al., 1978) and thus the concentration of this peptide may be higher in food preparations containing blood-based binding agent. This may apply more to bovine fibrinogen compared to porcine fibrinogen since both fibrinopeptides A and B were detected in foods spiked with porcine-derived binding agent. It is also possible that matrix components may interfere with the detection of bovine fibrinopeptide A, for example due to interstitial enzymes in meats.

3.2. Collaborative study

It was desirable that the collaborative study should challenge the method as much as possible in order to maximise the output of the trial. Further, it was desirable to include as many identical samples as possible in order to gain the highest number of samples \((n)\) and thus the most credible statistical analysis. There was, due to financial constraints, a limit to the number of samples which could be analysed at each participating laboratory. Since, as described above, it appeared more challenging to identify bovine fibrinopeptides compared to porcine with fewer transitions reported, it was decided to use samples spiked with bovine-derived binding agent in the trial. Therefore, if the participants could possibly identify the addition of the binding agent which had proved to be the most challenging in Round 1, it could be inferred that there was a strong possibility that the method would be applicable to screen for foods containing porcine binding agent.

During the inter-laboratory trial, all forty three test materials were correctly identified as ‘bovine fibrinopeptides detected’ and ‘porcine fibrinopeptides not detected’. Again, all laboratories met the quality assurance criteria. Example LC–MS/MS chromatograms are shown in Fig. 2.

It was known before the trial that food matrices can suppress the signal related to bovine fibrinopeptide A (Grundy et al., 2007). Therefore, depending on instrument sensitivity, it was expected that participants may only detect a single transition of bovine fibrinopeptide A for samples containing bovine blood-based binding agent. Four of the five participants were in fact able to confirm the presence of bovine fibrinopeptides in the test materials, observing two relevant transitions for fibrinopeptide A. One participant observed only one transition (bovine fibrinopeptide A, 946.4 > 695.0). This transition eluted at the expected retention time compared to the relevant reference standard, and thus this (and all other) QA criteria for reporting (but not confirming) detection were met. The instrument used by this participating laboratory is deemed the least sensitive of the instruments involved in the trial (Quattro Pt MS, Waters Micromass) and these results are attributed to this lower sensitivity.

3.3. Statistical analysis

The method used is a qualitative method. The statistical analysis performed was an estimate of the binomial confidence interval according to the modified Jeffries interval, as described by Brown, Cai, and DasGupta (2001). This is the approach recommended in Macarthur and von Holst (2012) in cases where 100% positive or negative results are observed in a study.

Analysing the data from the collaborative trial by this approach, the false positive and false negative rates of the method were estimated as less than 10% on average across the laboratories. The false negative rate is also estimated as less than 10% on average across the laboratories. Given that bovine fibrinopeptides were (correctly) detected above the limit of detection in all forty three test materials and porcine fibrinopeptides were (correctly) not detected above the LOD in these test materials, the results generated to date are consistent with a false positive and false negative rate of 0%. As more samples are analysed, this figure would be expected to increase.

3.4. Future work

The method under investigation is a qualitative method. With the advent of more sensitive triple quadrupole instruments since the method was originally developed, it is believed that this method may, with some optimisation, perform in a quantitative manner. This prospect would be advantageous, firstly, since quantitative data provides better validation data for a method. Secondly, quantitative methods provide more performance information for accurately calculating false positive and negative rates and accrual of such data is beneficial in terms of assessing the performance of the technique. Finally, quantitative data enables differentiation between adventitious presence and deliberate
Fig. 1. Example ESI mass spectra showing products of bovine and porcine fibrinopeptide standards generated by a participant using a TSQ Quantum Ultra MS (Thermo Scientific). (i) Bovine fibrinopeptide A (946.8 > 694.8 and 227.1), (ii) Bovine fibrinopeptide B (783.0 > 797.5 and 296.1), (iii) Porcine fibrinopeptide A (881.4 > 1091.5 and 201.1) and (iv) Porcine fibrinopeptide B (734.0 > 1008.5 and 157.1).
addition of an analyte. It is therefore intended to assess the suitability of the method for quantitative interrogations. Further, to increase the scope and potential of the method, it is planned to validate the technique against commercial meat products.

4. Conclusions

It has been demonstrated that an LC–MS/MS screening method for the addition of bovine and porcine blood-based binding agents to foods can be successfully transferred to other operators located in different food testing laboratories and using a range of different triple quadrupole instruments. During the trial, the species origin of binding agent which had been added to foods at a concentration of only 10% (v/w) was correctly assigned for all forty three test materials. Using an estimate of the binomial confidence interval according to the modified Jeffreys interval (Brown et al., 2001), given the number of samples tested, both the false positive and false negative rates of the method were determined as less than 10%. Further, since all samples were correctly identified, the results of the study were consistent with a false positive and false negative rate of 0%.

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