Complete mapping of disulfide linkages for etanercept products by multi-enzyme digestion coupled with LC-MS/MS using multi-fragmentations including CID and ETD

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Abstract

The disulfide linkages of two etanercept products, Enbrel® (innovator drug) and TuNEX®, were characterized and compared using a multi-fragmentation approach consisting of electron transfer dissociation (ETD) and collision induced dissociation (CID) in combination with multi-enzyme digestion protocols (from Lys-C, trypsin, Glu-C, and PNGase F). Multi-fragmentation approach allowed multi-disulfide linkages contained in a peptide to be un-ambiguously assigned based on the cleavage of both the disulfide and the backbone linkages in a MS3 schedule. New insights gained using this approach were discussed. A total of 29 disulfides, Cys18-Cys31, Cys32-Cys45, Cys35-Cys53, Cys56-Cys71, Cys74-Cys88, Cys78-Cys-96, Cys98-Cys104, Cys112-Cys121, Cys115-Cys139, Cys-142-Cys157, Cys163-Cys178 in TNFR portion and Cys240-Cys240, Cys246-Cys246, Cys249-Cys249, Cys281-Cys341, Cys387-Cys445 in IgG1 Fc domain, were completely assigned with the demonstration of the same disulfide linkages between the Enbrel® and TuNEX® products. The data showed the higher order structure was preserved throughout the recombinant manufacturing processes and consistent between the two products.

1. Introduction

Disulfide linkages are directly involved in appropriate protein conformations and therefore have a significant influence on protein functions. As a result, regulatory agencies expect disulfide connectivity to be determined as part of product characterization [1,2]. Etanercept is a fusion protein that binds strongly to soluble tumor necrosis factor alpha (TNF-α). It
consists of a recombinant human TNF receptor 2 bound to the Fc portion of an immunoglobulin, and was the first TNF-α antagonist approved in 1998 for moderate to severe plaque psoriasis, psoriatic arthritis, ankylosing spondylitis and rheumatoid arthritis [3,4]. Except complicated N/O-glycan forms in the hinge and Fc region [5,6], etanercept molecule contains thirteen intra-chain and three inter-chain disulfide bonds, the majority of which are located in the TNF receptor region of the molecule [2,7]. Such complicated disulfide bridging in etanercept represents a new quality attribute that is critical for biopharmaceutical functionality, and should thus be carefully monitored and controlled to guarantee patient safety [7].

Mapping highly complicated disulfide linkages such as etanercept is challenging. Currently, different strategies have been used to identify disulfide bonds like comparison of LC-MS/MS between a reduced and non-reduced protein digest [8–13]. Partial reduction and differential alkylation [14–17] or chemical labeling [18,19] have been applied in sample preparation to help the analysis of the disulfide bonds. Collision-induced dissociation (CID) in LC-MS is the most common means of fragmentation to derive polypeptide structure information [20–23]. However, modifications such as disulfide bonds are not typically fragmented by CID [22,24–26]. A triple digestion method has been utilized to map the disulfide linkages of etanercept by reducing the number of linkages of a native peptide digest such that the linkage could be assigned to the only two remaining cysteine residues on the peptide [26,27]. Nevertheless, with only limited or no peptide backbone sequence information gained by CID, it is hard to confirm the sequences that were linked by disulfides.

Alternatively, electron-transfer dissociation (ETD) has been shown to break disulfide linkages and allowed the linked sequences to be assigned unambiguously via MS3 [28,29]. Our goal is thus to use modern ETD technique coupled with ETD/CID method to characterize both the innovator etanercept drug (Enbrel®) and its biosimilar (TuNEX®). Results derived from this study using multiple fragmentations including ETD and CID were compared to that reported by using CID alone.

2. Experimental

2.1. Samples

TuNEX® was produced by Chinese Hamster Ovary (CHO) cells as a dimeric, secreted, soluble protein. After collection for several days, the expressed protein was purified by a sequential downstream process including several validated r-protein A chromatographic, ion-exchange chromatographic and ultrafiltration steps combined with viral filtration. The TuNEX® lot# 02F09 was provided as 1.0 mg/mL × 6 mL for characterization. Enbrel®, lot# 02S03, was obtained from Pfizer, and provided as 1.0 mg/mL × 2 mL. Each sample was aliquoted as 50 µL per vial (50 µg) and stored at −80 °C before analysis.

2.2. Reagents

Sequencing-grade trypsin was purchased from Promega (Madison, WI, USA). Achromobacter protease I (Lys-C) and Glu-C were obtained from Roche (Nutley, NJ, USA). PNGase F, ammonium bicarbonate (NH4HCO3), and formic acid (FA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). LC-MS grade acetonitrile (ACN) was purchased from J. T Baker (Phillipsburg, NJ, USA). Amicon centrifugal filter (10 kDa MWCO) was obtained from Millipore (Bedford, MA, USA), and HPLC water was deionized to 18 MΩ by a Millipore Milli-Q system.

2.3. Enzymatic digestion

Protein solution (1.0 mg/mL of 100 µL) was buffer exchanged with 100 mM ammonium bicarbonate (pH 8 or pH 6.8) using a 10 kDa molecular weight cutoff filter to a concentration of 1 mg/mL (100 µL). In order to assess potential disulfide scrambling, in addition to pH 8, a slightly less than alkaline pH (pH 6.8) was also used in the enzymatic digestion protocol to examine whether any alternative disulfide linkages could be observed from the two different pH digestion conditions [20]. For trypsin digestion, trypsin (1:50, w/w) was added to the protein solution at room temperature for 8 h and then added a second time (1:50, w/w) for 12 h at room temperature. For Lys-C digestion, the endoprotease Lys-C (1:50, w/w) was added to the protein solution for 4 h at 37 °C. For Lys-C plus trypsin digestion, the protein solution was added with endoprotease Lys-C (1:50 w/w) and trypsin (1:50 w/w) for 24 h at room temperature. For Lys-C plus trypsin plus Glu-C digestion, the protein solution was added with endoprotease Lys-C (1:50 w/w), trypsin (1:50 w/w), and Glu-C (1:20 w/w) for 24 h at room temperature. Similarly, for additional PNGase F digestion, PNGase F (10 units/mg) was added to the above enzyme solution for 24 h at room temperature. In all cases, digestion was terminated by addition of 1% formic acid. An aliquot of 2 µg of the enzyme digest was analyzed per LC-MS run.

2.4. LC-MS

An Agilent 1200 nano-HPLC system (Agilent, Santa Clara, CA, USA) with a Agilent pre-column (300 µm × 5 mm, 5 µm C18), and an analytical column (75 µm i.d. × 15 cm, 3 µm C18) was coupled online to an LTQ-Orbitrap-ETD XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). Mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile) were used for the gradient consisted of (i) 5 min at 2% B for sample loading, (ii) linear from 2% to 40% B over 40 min, (iii) linear from 40% to 80% B over 10 min, and finally (iv) isocratic at 80% B for 10 min. Flow rate was maintained at 1 µL/min for the pre-column and 200 nL/min for the analytical column. The LTQ-Orbitrap-ETD XL mass spectrometer was operated in the data-dependent mode to switch automatically between MS (scan 1 at the Orbitrap), CID-MS2 (scan 2 at the LTO), and ETD-MS2 (scan 3 at the LTO). Briefly, after a survey MS spectrum from m/z 300 to m/z 2000, subsequent CID-MS2 and ETD-MS2 steps were performed on the same precursor ion with a ±2.5 m/z isolation width. Any inadequate information (assignment) in the CID-MS2 and ETD-MS2 spectra will be repeated by targeting the desired ions, e.g. the same precursor but with different charge state, in order to gain additional linkage information. These targeted approach will be repeated (e.g. targeting multiple charges of a precursor ion or the same disulfide-linked peptide but with
Fig. 1 — (A) Primary sequence and sites of disulfide linkages of TuNEX®. (B) Disulfide linkages of Etanercept.
different enzymatic cleavages or mis-cleavages) until the linkage information was adequate. In addition, a targeted CID-MS² after ETD for the ions of interest was further performed as necessary.

2.5. Disulfide assignment

The anticipated disulfide-linked tryptic or multi-enzyme digested peptide masses with different charges were first calculated using free software (http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msproduct), and then matched to the observed masses in the LC-MS chromatogram. The matched masses (with <5 ppm mass accuracy) were further confirmed by the corresponding CID-MS² and ETD-MS² fragmentation manually, as well as by CID-MS³ fragmentation, as needed.

3. Results and discussion

The fusion antibody (Enbrel® or TuNEX®) contains two identical pairs of monomers, which are connected by inter-molecular and intra-molecular disulfides. The only obvious difference between the two products was a two-AA residue variance in the heavy chain (Fc) [30]. This, however, was not expected to affect the disulfide linkages. For illustration, the primary structure of TuNEX® with the sites of expected disulfide linkages is shown in Fig. 1A. For each monomer, there are two domains consisting of TNFR and IgG1 Fc (Fig. 1B). The TNFR domain contains only intra-molecular disulfides, a total of 22 disulfides, derived from the 11 intra-molecular disulfides multiply by 2 (2 identical monomers). The IgG1 Fc domain contains 4 intra-molecular disulfides (multiply by 2 equals 4).

Table 1A – Summary of disulfide linkages identification at TNFR of Etanercept for both Enbrel® and TuNEX®.

| #<sup>a</sup> | Site | Cys<sup>ω</sup> status | Enzyme + trypsin + Glu-C<sup>ω</sup> | Corresponding peptide sequence | I.D.<sup>ω</sup> (method) | Embrel® (observed) | TuNEX® (observed)<sup>ω</sup> |
|---|---|---|---|---|---|---|---|
| 1<sup>a</sup> | Cys18-Cys31<sup>ω</sup> Cys32-Cys45<sup>ω</sup> | 2 disulfide linkages<sup>ω</sup> | Lys-C + trypsin + Glu-C<sup>ω</sup> | PGSTCR (14-19)<sup>ω</sup> YYDQTAQMCSCSK (23-34)<sup>ω</sup> VFCTK (43-47)<sup>ω</sup> | CID and ETD<sup>ω</sup> | Yes<sup>ω</sup> 34.29 min<sup>ω</sup> | Yes<sup>ω</sup> 34.23 min<sup>ω</sup> |
| 2<sup>a</sup> | Cys35-Cys53<sup>ω</sup> Cys56-Cys71<sup>ω</sup> Cys74-Cys88<sup>ω</sup> | 3 disulfide linkages<sup>ω</sup> | Lys-C + trypsin + Glu-C<sup>ω</sup> | CSPQYHAK (35-42)<sup>ω</sup> TSDTVCDSE (48-57)<sup>ω</sup> CLSCGSR (71-77)<sup>ω</sup> TOACTR (85-90)<sup>ω</sup> | CID and ETD<sup>ω</sup> | Yes<sup>ω</sup> 19.69 min<sup>ω</sup> | Yes<sup>ω</sup> 19.62 min<sup>ω</sup> |
| 3<sup>a</sup> | Cys78-Cys96<sup>ω</sup> Cys98-Cys104<sup>ω</sup> | 2 disulfide linkages<sup>ω</sup> | Lys-C + trypsin + Glu-C<sup>ω</sup> | CSSDQVE (78-84)<sup>ω</sup> ICTCR (95-99)<sup>ω</sup> PGWVCALSX (100-108)<sup>ω</sup> | CID and ETD<sup>ω</sup> CID-MS²<sup>ω</sup> | Yes<sup>ω</sup> 32.26 min<sup>ω</sup> | Yes<sup>ω</sup> 31.90 min<sup>ω</sup> |
| 4<sup>a</sup> | Cys112-Cys121<sup>ω</sup> | 1 disulfide linkage<sup>ω</sup> | Lys-C + trypsin<sup>ω</sup> | QEGCR (109-113)<sup>ω</sup> CRPGFVAR (121-129)<sup>ω</sup> | CID<sup>ω</sup> | Yes<sup>ω</sup> 22.33 min<sup>ω</sup> | Yes<sup>ω</sup> 22.65 min<sup>ω</sup> |
| 5<sup>a</sup> | Cys115-Cys139<sup>ω</sup> | 1 disulfide linkage<sup>ω</sup> | Lys-C + trypsin<sup>ω</sup> | LACLR (114-119)<sup>ω</sup> TSDVVCK (134-140)<sup>ω</sup> | CID and ETD<sup>ω</sup> | Yes<sup>ω</sup> 26.92 min<sup>ω</sup> | Yes<sup>ω</sup> 27.47 min<sup>ω</sup> |
| 6<sup>a</sup> | Cys142-Cys157<sup>ω</sup> | 1 disulfide linkage<sup>ω</sup> | Lys-C + trypsin + PNGase<sup>ω</sup> | PCAGTFSDDTSTSDICR (141-158)<sup>ω</sup> | CID<sup>ω</sup> | Yes<sup>ω</sup> 30.42 min<sup>ω</sup> | Yes<sup>ω</sup> 30.61 min<sup>ω</sup> |
| 7<sup>a</sup> | Cys163-Cys178<sup>ω</sup> | 1 disulfide linkage<sup>ω</sup> | Lys-C + trypsin + PNGase<sup>ω</sup> | PHQICNVVAIPGDASMDAVC TSTS PTR (159-185)<sup>ω</sup> | CID<sup>ω</sup> | Yes<sup>ω</sup> 36.64 min<sup>ω</sup> | Yes<sup>ω</sup> 37.12 min<sup>ω</sup> |

Note: Disulfide linkages are connected by red lines. The N-glycosylation motifs are underlined, in which the N (Asn) converts to D (Asp) after deglycosylation by PNGase F.
and 3 inter-molecular disulfides, a total of 7 disulfides. Thus, etanercept was expected to contain a total of 29 disulfides, as shown in Fig. 1B.

For such a complicated disulfide structure, a proper enzyme digestion strategy is needed. In principle, identification of a single disulfide linkage is straightforward because there is usually only one possibility for connection. Consequently, enzymes, which can cut the protein to the peptide size containing only single disulfide, are desired. However, the intertwined disulfides or the protein’s backbone sequence may prevent enzyme digestion to the desired peptide size. In addition, peptide sizes are preferred to be 1–5 kDa. Recovery and electrospray ionization efficiency can be a problem for larger peptides, while smaller peptides may not retain well on a typical reversed phase column. Thus, selection of proper enzymes or multiple enzymes should be considered for the size adjustment. Also, for the disulfide-linked peptides containing N-glycosylation, additional PNGase F digestion needs to be considered to remove the N-linked glycans in order to reduce complexity. After surveying several enzyme combinations, a cocktail protocol, which combined Lys-C plus trypsin or Lys-C plus trypsin plus Glu-C, was found to work effectively for most of the disulfide analysis. With additional PNGase F, the disulfide analysis was successful for the peptides with N-glycosylation as well. It should be noted that enzymatic cleavages of the protein are the same by using either trypsin or Lys-C plus trypsin. Nevertheless, the use of Lys-C plus trypsin seems to yield slightly higher digestion efficiency than trypsin alone. Similarly, the use of PNGase F enzyme would not yield additional cleavages except the deglycosylation of N-glycosylated sites. The addition of PNGase F, even for the disulfides without N-glycosylation, was used to improve the digestion efficiency as needed.

Table 1A lists the identified disulfide linkages of Enbrel® and TuNEX® from TNFR portion, respectively. Table 1B lists the identified disulfide linkages of Enbrel® and TuNEX® from IgG1 Fc domain, respectively. The linkage sites, corresponding peptide sequences, digest enzymes, identification methods by mass spectrometry, and observed positions in LC-MS analysis of Enbrel® and TuNEX® are summarized in the table. The detailed identification is described in the following text.

For the peptide with the three disulfides (Cys35-Cys53, Cys56-Cys71, and Cys74-Cys88, #2 in Table 1A), the same digestion protocol and identification methods were used. Again, indistinguishable mass (Fig. 2A,B) and fragmentation patterns (Fig. 2C–F) were observed for both Enbrel® and TuNEX® in this disulfide-linked peptide. For the linkage assignment, the CID fragmentation of the same disulfide-linked peptide but with different charge states were shown, i.e. m/z 657.67 of 5+ (Fig. 2C,D) and m/z 1095.74 of 3+ (Fig. 2E,F), with the identification of the same cleavage for disulfide linkage sites (i.e. y5 on P3 peptide). The fragmentation from the two different charge states was used to prove that the site assignment was correct not only with respect to the cleavages but also with the consistency. All these information provided strong evidence again that both products have the same disulfides as shown.

Similarly, for the peptide with the two disulfides (Cys78-Cys96 and Cys98-Cys104, #3 in Table 1A), the digestion protocol required 3 enzymes (the combination of Lys-C plus trypsin plus Glu-C). This cocktail digestion protocol cut the disulfide-linked peptide to a proper size effectively for fragmentation by mass spectrometry. As shown in Fig. 3A (Enbrel®) and 3B (TuNEX®), the corresponding mass and charge of the precursor ion from both products and were illustrated with the accurate monoisotopic mass within 1 ppm for both products (i.e. m/z 596.0067 for Enbrel® and m/z 596.0065 for TuNEX® at 4+ charge state). In the figure, both monoisotopic masses also accurately matched the theoretical

| # | Site | Cys status | Enzyme | Corresponding peptide sequence | I.D. (method) | Enbrel® (observed) | TuNEX® (observed) |
|---|---|---|---|---|---|---|---|
| 8 | Cys240-Cys240, Cys246-Cys246, Cys249-Cys249 | 3 disulfide linkages | Lys-C + trypsin + Glu-C | SCDKHTCPPCPEAPE (239-253) | CID and ETD | Yes | Yes |
| 9 | Cys246-Cys246, Cys249-Cys249 | 2 disulfide linkages | Lys-C + trypsin + Glu-C | THICPPEAPE (243-253) | CID and ETD | Yes | Yes |
| 10 | Cys281-Cys341 | 1 disulfide linkage | Lys-C + trypsin | VTCVVVDVHEDPEVK (279-294) | CID | Yes | Yes |
| 11 | Cys387-Cys445 | 1 disulfide linkage | Lys-C + trypsin + Glu-C | WQQGNVFSCVMHE (437-450) | CID | Yes | Yes |
Fig. 2 – Mass and charge of Lys-C + trypsin + Glu-C-digested peptide with the three disulfides (Cys35-Cys53, Cys56-Cys71, and Cys74-Cys88), for Enbrel® (A) and TuNEX® (B). The sequence and theoretical mass of the peptide along with the observed monoisotopic (1st isotopic) mass are indicated in the insert. CID-MS² spectrum of the precursor for Enbrel® (C) and TuNEX® (D). CID-MS² spectrum of the precursor but with a different charge state (3+) from Figure 2A, B for Enbrel® (E) and TuNEX® (F). ETD-MS² spectrum of the precursor for Enbrel® (G) and TuNEX® (H).
Fig. 2 — (continued).
Fig. 3 – Mass and charge of Lys-C + trypsin + Glu-C-digested peptide with the two disulfides (Cys78-Cys96 and Cys98-Cys104), for Enbrel® (A) and TuNEX® (B). The sequence and theoretical mass of the peptide along with the observed monoisotopic (1st isotopic) mass are indicated in the insert. CID-MS² spectrum of the precursor for Enbrel® (C) and TuNEX® (D). ETD-MS² spectrum (using Orbitrap) of the precursor for Enbrel® (E) and TuNEX® (F). CID-MS³ spectrum of the precursor from Figure 3E (m/z 680.27) for Enbrel®(G) and Figure 3F (m/z 680.27) for TuNEX®(H).
Fig. 3 – (continued).
monoisotopic mass (m/z 596.0062), which was the peptide with the two disulfides (a loss of 4H from the backbone sequence). The disulfide linkage information was obtained by CID-MS² and ETD-MS² of the precursor ion, as illustrated in Fig. 3C,F (Enbrel®), as well as Fig. 3D,F (TuNEX®), and by CID-MS³ of the precursor after ETD-MS². In the linkage assignment, the corresponding CID-MS² spectrum provided the sequence information (b ions). In addition to CID-MS², the corresponding ETD-MS² confirmed that the three peptides were linked together (the observation of P₁, P₂, and P₃ peptides), and confirmed further by the partially disulfide-dissociated peptides (i.e. P₁ + P₂ and P₂ + P₃). As shown by ETD (Fig. 3E,F), the linked peptides were P₁ with P₂, and P₂ with P₃, but not P₁ with P₃. We chose m/z 680.27 of 2+ (Fig. 3E,F) to do CID-MS³ (Fig. 3G,H). The CID-MS³ provided us the information of disulfide linkage (y3 on P₂ peptide). The fragmentation was used to prove Cys78-Cys96. And the remaining Cys98 should link Cys104. It should be noted that fragmentation was used to prove Cys78-Cys96. And the information of disulfide linkage (y3 on P₂ peptide). The dissociated peptides (i.e. P₁, P₂, and P₃ peptides), and confirmed further by the partially disulfide-dissociated peptides (i.e. P₁ + P₂ and P₂ + P₃). As shown by ETD (Fig. 3E,F), the linked peptides were P₁ with P₂, and P₂ with P₃, but not P₁ with P₃. We chose m/z 680.27 of 2+ (Fig. 3E,F) to do CID-MS³ (Fig. 3G,H). The CID-MS³ provided us the information of disulfide linkage (y3 on P₂ peptide). The fragmentation was used to prove Cys78-Cys96. And the remaining Cys98 should link Cys104. It should be noted that both Enbrel® and TuNEX® were demonstrated with indistinguishable precursor ion mass and fragmentation pattern by both CID and ETD spectra, which provided strong evidence that both products have the same disulfides in this linkage.

Using the same approach, the rest of disulfide linkages were identified as shown from Figs. S1–S9, corresponding to the disulfides from #1 and #4 to #11 in Tables 1A and 1B, respectively. Again, indistinguishable mass and consistent fragmentation patterns were observed between the two products.

The peptides with a single disulfide, the assignment was straightforward (only one connection possible) and thus only CID-MS² spectrum was sufficient and shown. The disulfides in the IgG1-Fc region, in which the linkages were known and conserved, the accurate mass and consistent pattern between Enbrel® and TuNEX® were shown for identification. In these cases, we have successfully mapped disulfide linkages using online LC-MS with ETD based on the favorable breakage of the disulfide bond. The cleaved or partially cleaved disulfides were further fragmented by CID-MS³ to obtain specific linkage locations.

Compared to the previous report using HCD alone [7], three disulfide linkages appear to differ: Cys98-Cys104 differed from the reported Cys98-Cys115; Cys112-Cys121 differed from the reported Cys104-Cys112; Cys115-Cys139 differed from the reported Cys121-Cys139. Two of them, Cys104-Cys112 and Cys121-Cys139 linkages, were previously mapped by precursors with two or more disulfide linkages. Because of the lack of critical fragments to map multi-disulfide linkages using CID alone, their assignments were based on X-ray data derived from TNF binding protein rather than etanercept products. As shown in Table 1A, Cys98-Cys104 and Cys78-Cys96 were assigned here by the same precursor using ETD to break the disulfide bonds and further fragmented by CID-MS³ (Fig. 3G,H). Furthermore, the Cys112-Cys121 (Supplement Fig. S2B) and Cys115-Cys139 (Supplement Fig. S3B) were assigned here by peptides with single disulfide linkage (Table 1A) using our multi-enzyme protocol. We have further investigated our data based on manual extraction. The theoretical ions (m/z) with the previously reported Cys104-Cys112 linkage were not detected here from either Enbrel® or TuNEX®. Furthermore, previous method using HCD alone has revealed four disulfide scrambling, Cys18-Cys74, Cys71-Cys88, Cys71-Cys74, Cys78-Cys88 among which, the percentage of Cys78-Cys88 scrambling was reported to be as large as 10%. However, only a much lower and insignificant percentage (<1%) of scrambling on Cys71-Cys74 or Cys78-Cys88 was detected from Enbrel® or TuNEX® here. In addition, we have checked alternative linkages such as Cys71-Cys98/Cys96-Cys104 and could not find significant signals for fragments generated by such linkages.

4. Conclusions

In summary, a LC-MS protocol using multi-enzyme coupled with multi-fragmentation approach have been employed to assign the status of all entire disulfide linkages including TNFR portion (22 disulfides) and IgG1 Fc domain (7 disulfides) of two etanercept (Enbrel® and TuNEX®) products. Compared to previous reports using HCD fragmentation alone, our method provided more straight forwards and un-ambiguous assignments for disulfide linkages. Based on repetitive analyses of specific lots of the two etanercept products, our data showed the higher order structure of the two products was consistent.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jfda.2018.11.007.

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