Proteomics—Tenderness: Application of Proteomic Tools to Define Contributions of Protein to Variation in Meat Tenderness

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INTRODUCTION

Tenderness is one of the attributes of fresh meat that is most commonly cited as being extremely important to consumer satisfaction with the product (Mennecke et al., 2007). Therefore, predicting and managing tenderness is important if processors and members of the industry are to maximize profits. Tenderness, however, has proven to be a very elusive trait to predict and manage. One procedure that is known to improve tenderness is aging. Yet even aging is not effective in all muscles or for all carcasses. One reason for the heterogeneity in the aging response is the underlying biochemistry of the various muscles, especially with respect to proteolysis of key proteins. Tenderization during the aging period is ultimately dependent on the activity of proteolytic enzymes within the muscle and muscle cells (Huff-Lonergan et al., 1996). Knowing this fact, however, has not made predicting the tenderization response any easier. The activity of the inherent enzymes is influenced by tissue pH, temperature, and posttranslational modifications of the enzymes and their inhibitors (Huff-Lonergan et al., 2010). In addition, the substrates of the enzymes may be affected by these same factors, potentially altering their susceptibility to being degraded. As a result, unraveling the story of postmortem tenderization of meat has proven to be quite elusive.

As advances in biochemical techniques for studying muscle and meat are made, the research community is continually expanding its capability to explore more deeply the intricate interrelationships among proteolytic enzymes, their substrates, and tissue and cellular environmental conditions that govern the rate and extent of tenderization achieved by different muscles. One collection of techniques that is being used to understand differences in muscles and meat with respect to tenderness is proteomics (Bendixen et al., 2011). Proteomics is a broad term that encompasses a wide variety of techniques that aim to examine and characterize the entire protein profile of the cell or tissue (referred to as the proteome). Proteomic tools can provide insight into the relative abundance of specific proteins, protein modifications (including proteolysis, phosphorylation, and glycosylation), subcellular localization of specific proteins, interaction partners, and protein sequence information. It is important to understand, however, that the differences noted are all with respect to a particular point in time. The very nature of the proteome dictates this because even in postmortem muscle, the proteome is dynamic.

Proteome analysis has a distinct advantage over DNA or mRNA-based analysis techniques because it does give a reflection of the actual protein profile and state of the proteins in a tissue at a particular point in time, something that DNA or mRNA analyses cannot do. Additionally, proteomics can give information on posttranslational modification of proteins, something DNA-based techniques are also not equipped to do. In its broadest sense, proteomics attempts to provide researchers with cellular protein expression data and can help explain how protein profiles influence observed differences in cells, tissues, or meat (Mullen et al., 2009).

Proteomics is very powerful in helping understand the role of proteins in various tissues or cells. It is, however, a challenging tool to use if the desired outcome is truly to identify the entire complement of proteins in a cell. Investigators should focus first on their research question before commencing any experiment, but this might be even more important in the "global" experiments that use any of the new integrated "omics" approaches. Often, researchers will choose to narrow their focus and look more specifically at a particular organelle or subcellular

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fraction. This decision cannot be made lightly, and interpretation of the results must take into consideration the techniques used as well as all the steps involved in sample preparation.

TECHNIQUES

Many techniques in protein chemistry that have existed for years (chromatography, electrophoresis, immunoblotting) are part of the proteomic tools that we use today. Two major developments have driven a quantum leap in the power of these tools: 1) application of mass spectroscopy technology to determine the identity of proteins (Mirza and Olivier, 2008), and 2) the development and maintenance of protein databases for rapid searches and investigations. The most frequently used approaches used in experiments have been electrophoresis methods, and these are briefly described here.

2-Dimensional Gel Electrophoresis

One of the most common techniques used to begin to examine the proteome is 2-dimensional gel electrophoresis (**2DE**). Because the proteome of skeletal muscle contains many thousands of proteins, this technique is a good starting point. Two-dimensional gel electrophoresis provides good resolution of proteins in a complex mixture and has an advantage over 1-dimensional electrophoresis methods because it can provide information not only on the relative molecular weight of a protein, but also on its isoelectric point. The resolution attainable from 2DE is largely because the separation takes place in 2 dimensions. The first dimension uses an electrophoresis gel (typically in strip form) that separates the proteins on the basis of their isoelectric point. The degree of separation achieved is dependent on the width of the pH range chosen and the length of the strip. The second dimension is based on separation by molecular weight (Gorg et al., 2000). Thus, the advantage that 2DE brings is that it can separate proteins having similar molecular weights but different isoelectric points. A good example of this is the separation of phosphorylated isoforms of the same protein. In a 2DE gel, the phosphorylated form of the protein will migrate to a more acid region of the first-dimension gel than will the nonphosphorylated form of the protein. In general, the greater the amount of phosphorylation, the more acidic the protein will appear in the isoelectric focusing gel (Maurides et al., 1989). Because the phosphorylation will not significantly alter the molecular weight of the protein, the isoforms (both phosphorylated and nonphosphorylated forms) will migrate at the same molecular weight. Thus, if a protein with phosphorylated and nonphosphorylated isoforms is present, then the protein will appear as a horizontal chain in the 2DE gel.

Two-dimensional gel electrophoresis is not without its limitations. Because loading of the protein onto the first dimension (isoelectric focusing) gel is passive, usually through rehydration, very large proteins may be excluded from the analysis. In addition, very acidic and very basic proteins can be more difficult to resolve in the first dimension (Gorg et al., 2000). Two-dimensional gel electrophoresis may also be limited in the number of proteins that can be resolved, and highly abundant proteins may be overrepresented in the proteome (Gorg et al., 2000). This can be overcome by prefractionating the sample by separating the sarcoplasmic and myofibrillar fractions, for example, or by selectively removing abundant proteins; using an antibody column to adsorb albumin in blood serum samples, as an example (Gorg et al., 2000; Bianchi et al., 2007; Restuccia et al., 2009; Marco-Ramell and Bassols, 2010; Righetti and Boschetti, 2010).

Difference Gel Electrophoresis

One limitation of 2-dimensional (**2-D**) gels is that typically only 1 sample per gel may be run. This makes comparisons between samples very time-consuming and laborious. Generally, when investigators want to make comparisons, they must run many gels of the same samples. This requires many multiple runs of gels to be compared (Gorg et al., 2000).

A modification of 2DE is 2-D fluorescence difference gel electrophoresis (**DIGE**). This technique can be used to detect changes in protein profile, abundance, or both in the fractions of muscle from 2 different samples at the same time. This analysis uses novel fluorescent dyes (CyDye DIGE Fluor saturation dyes, Amersham Biosciences, Piscataway, NJ) specifically to label proteins from individual samples and to provide the sensitivity needed to quantify minute differences in protein abundance in a 2-D gel format. The dyes are matched in mass and have a net zero charge, allowing the labeled proteins to migrate to the same position in 2-D gels. These dyes are spectrally resolvable, allowing multiplexing of samples within a single gel. In an experiment, individual fractions of proteins from 2 experimental samples and a reference sample (pooled sample composed of all samples in the experiment) are labeled with different CyDye Fluors and run on 2-D PAGE gels (Marouga et al., 2005). The different samples are then imaged using excitation wavelengths that will cause the proteins labeled with a specific dye to be imaged. The images can then be merged and protein differences can be viewed (Figure 1).

Diagonal PAGE

Two-dimensional sequential nonreducing and reducing SDS-PAGE (diagonal PAGE; Sommer and Traut, 1974) is an approach used to identify proteins that have formed intermolecular disulfide bonds. As the name implies, this method uses a first dimension of electrophoretic separation (nonreducing), followed by a second dimension that includes a reducing step. If the reducing step has caused a release of an intermolecular disulfide bond, the protein partners in that bond will migrate off the diagonal in the second dimension.

Regardless of the electrophoresis method used, protein spots that are identified as being significantly different are

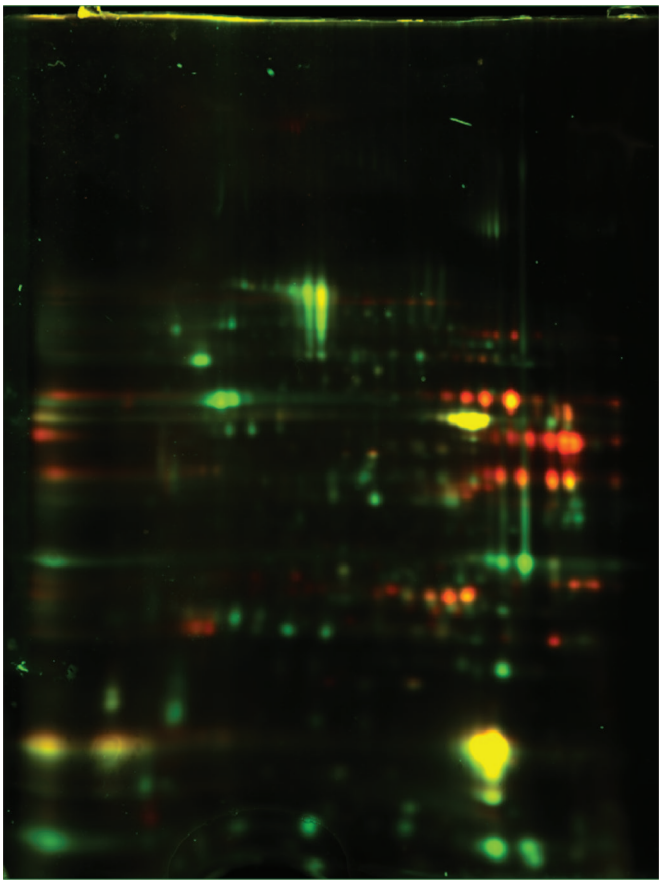

Figure 1. Two-dimensional fluorescence difference gel electrophoresis image of sarcoplasmic proteins from longissimus dorsi (LD) and vastus intermedius (VI). Red indicates that proteins were found only in LD. Green indicates that proteins were found only in VI. Yellow indicates that proteins were found in both LD and VI.

"picked" or removed from the gel and are used for protein mass fingerprinting. This technique analyzes picked proteins that have been digested (typically with trypsin) and that are subjected to mass spectrometry to ascertain the identity of the proteins by comparing the sequence obtained from the digested protein with sequences of known proteins in a database.

USE OF PROTEOMICS TO IDENTIFY PROTEINS INVOLVED IN TENDERIZATION

Because the protein component of meat is the primary contributor to differences in tenderness and because changes in the protein profile during aging affect tenderness, proteomic approaches are quite useful in investigations designed to understand meat tenderness. One of the major enzyme systems that is involved in the postmortem tenderization of meat is the calpain system (Huff-Lonergan and Lonergan, 1999; Huff Lonergan et al., 2010). Calpains are a family of 14 related enzyme isoforms. The isoforms μ-calpain and m-calpain are the most well characterized of the known family members (Goll et al., 2003). These 2 isoforms are ubiquitous and are calcium dependent. In addition to calcium, μ- and m-calpain are regulated by their endogenous inhibitor calpastatin, pH, autolysis, and potentially by other factors that have been suggested, including phospholipids and phosphorylation, oxidation, and nitrosylation (Goll et al., 2003; Melody et al., 2004; Rowe et al., 2004; Maddock et al., 2005; Carlin et al., 2006; Bee et al., 2007; Huff Lonergan et al., 2010). In fact, oxidation of μ-calpain reversibly inhibits the proteinase by forming an intramolecular disulfide bond (Lametsch et al., 2008)

It is important to know what protein "partners" other than calpastatin are present in skeletal muscle that may affect the activity of these enzymes. Proteomic techniques have recently been used in several studies to aid in identifying some of these potential interacting proteins. Recently, by using evidence gathered from a combination of immunoprecipitation, electrophoresis, and mass spectroscopy techniques, researchers have identified proteins that may be associated with the calpains in muscle (Brulé et al., 2010). When the putative protein-binding partners for calpain were identified using this particular cadre of techniques, they revealed proteins that fell into 5 categories of proteins. These categories were 1) proteins involved in calcium homeostatis, 2) cytoskeletal proteins 3) sarcomeric proteins involved in contraction and myofibrillar stability, 4) mitochondrial proteins, and 5) proteins involved in the metabolism of glucose. A couple of the proteins involved in calcium homeostasis—the ryanodine receptor (calcium release channel) and the sarcoplasmic or endoplasmic calcium adenosine triphosphatase (SERCA1)—may be binding partners for the calpains. Additionally, the ryanodine receptor was shown to be a substrate for the calpains (Brulé et al., 2010). Given the fact that the calpains are calcium-requiring enzymes, these relationships with 2 of the major proteins involved in regulating calcium homeostasis are intriguing and could have implications for understanding the regulation of the postmortem activity of calpains and the subsequent development of meat tenderness.

Protein oxidation and aggregation during the conversion of muscle to meat as well as postmortem aging are known to cause toughening (Lund et al., 2007, 2011). Oxidative environments are also known to increase the force necessary to shear muscle fibers (Lund et al., 2008). It has been held that oxidation-induced aggregation of myosin heavy chain (Xiong et al., 2009) could contribute to this increase in toughness. Kim et al. (2010) used diagonal PAGE in combination with protein mass fingerprinting to confirm that myosin heavy chain was a primary partner in the aggregation of meat protein caused by oxidation. An important additional finding was that titin was cross-linked with myosin heavy chain in response to high-oxygen packaging (Kim et al., 2010). In another study, Brennan et al. (2004) used diagonal PAGE to suggest that myosin heavy chain is a target of disulfide formation in living muscle cells as well.

The use of the DIGE technique has proven to be very powerful in helping to determine what proteins may be altered in muscles that differ in tenderness. Two beef muscles that have been shown to differ greatly in their response to aging are the longissimus dorsi and the adductor. Although the longissimus dorsi typically undergoes a decline in shear force (increase in tenderness over 14 d of postmortem aging), the adductor has been shown not to change significantly over that time period (Anderson et al., 2011a). These characteristics make these muscles ideal candidates for examining postmortem changes in proteins that may be responsible for tenderization. In a recent study, 2D-DIGE was used to compare changes in the protein profile in the myofibrillar and sarcoplasmic fractions over storage time. In both the adductor and the longissimus dorsi, glyceraldehyde-3-phosphate dehydrogenase decreased in abundance in the sarcoplasmic fraction and increased in abundance in the myofibrillar fraction. Additionally, in the longissimus dorsi, the proteins actin, a fragment of myosin heavy chain 1, and myomesin-2 increased in abundance in the myofibrillar fraction during storage, whereas the protein α-actinin-3 increased in the sarcoplasmic fraction during the same storage period (Anderson, 2011). These proteins did not change over time in the fractions from the adductor—potentially indicating that alterations of actin, a fragment of myosin heavy chain 1, myomesin-2, and α-actinin-3 may prove to be fruitful targets when looking for proteins that could predict tenderization or at least help define mechanisms for tenderization.

Another protein that has been identified by 2D-DIGE as potentially being involved in meat tenderization is myosin light chain 1. In 2D-DIGE experiments using the sarcoplasmic fraction of beef longissimus dorsi from samples that were more tender, myosin light chain 1 was found to be more abundant than in the less tender samples (Anderson et al., 2011b). Additionally, myosin light chain 1 was shown to be released into the soluble fraction when myofibrils are incubated with highly purified μ-calpain (Anderson et al., 2011b). Myosin light chain 1 is associated with the head region of myosin, and it serves to regulate the myosin motor. In addition, it interacts with actin in the actomyosin complex, making it an intriguing candidate to consider when trying to identify proteins that may predict tenderness.

Proteomics, and more specifically, the 2D-DIGE tool, can also be useful in detecting posttranslational modifications of proteins that may be related to tenderness. One example of this is the potential differences that exist in the phosphorylation state of phosphoglucomutase 1. Isoforms of this protein are differentially expressed in beef samples that differ in tenderness (Bouley et al., 2005; Anderson et al., 2011b). In 2 separate studies, the most alkaline isoform of this protein was shown to be the most abundant in the less tender samples (Bouley et al., 2005; Anderson et al., 2011b). Additionally, Anderson et al. (2100b) took an extra step and confirmed, using a phosphoprotein stain (ProQ Diamond, Molecular Probes Inc., Eugene, OR), that indeed the difference in the isoforms was due to differences in phosphorylation state and that postmortem muscle that had the least amount of phosphorylation of phosphoglucomutase 1 was more abundant in the less tender samples (Anderson, 2011). Because phosphoglucomutase 1 is an important enzyme in glycogen metabolism and because phosphorylation can modulate its activity, further examination of this relationship is warranted.

SUMMARY

Proteomic tools provide a means to investigate pathways that are important in regulating the postmortem tenderization of meat. Identification of binding partners for key enzymes, minor degradation of contractile proteins that alters protein interactions, and discovery of the relationships that govern posttranslational modifications of key enzymes are among the many new discoveries that can be made when researchers use the newest technologies in the most appropriate fashion to answer the age-old question of what governs the postmortem tenderization process. Improvement of meat quality using proteomic approaches will continue to rely on the application of robust and sensitive quantification methods while increasing throughput of the methods. However, true advancement will require careful experimental design, collaboration, and sharing of data through public repositories.

REFERENCES

Anderson, M. J. 2011. Identification of proteins and biological processes associated with tenderness in beef muscles, Iowa State University, Ames.

Anderson, M. J., S. M. Lonergan, C. A. Fedler, K. Prusa, J. Binning, and E. Huff Lonergan. 2011a. Round muscle profiling: Management of tenderness and sensory improvement of specific muscles with aging. Meat Sci. (Submitted)

Anderson, M. J., S. M. Lonergan, and E. Huff Lonergan. 2011b. Myosin light chain 1 release from the myofibrillar fraction during postmortem aging is a potential indicator of proteolysis and improvement in tenderness of beef. Meat Sci. (Submitted)

Bee, G., A. L. Anderson, S. M. Lonergan, and E. Huff-Lonergan. 2007. Rate and extent of pH decline affect proteolysis of cytoskeletal proteins and water-holding capacity in pork. Meat Sci. 76:359–365.

Bendixen, E., M. Danielsen, K. Hollung, E. Gianazza, and I. Miller. 2011. Farm animal proteomics—A review. J. Proteomics 74:282–293.

Bianchi, L., P. Lorenzoni, L. Bini, E. Weber, C. Tani, A. Rossi, M. Agliano, V. Pallini, and G. Sacchi. 2007. Protein expression profiles of *Bos taurus* blood and lymphatic vessel endothelial cells. Proteomics 7:1600–1614.

Bouley, J., B. Meunier, C. Chambon, S. De Smet, J. F. Hocquette, and B. Picard. 2005. Proteomic analysis of bovine skeletal muscle hypertrophy. Proteomics 5:490–500.

Brennan, J. P., R. Wait, S. Begum, J. R. Bell, M. J. Dunn, and P. Eaton. 2004. Detection and mapping of widespread intermolecular protein disulfide formation during cardiac oxidative stress using proteomics with diagonal electrophoresis. J. Biol. Chem. 279:41352–41360.

Brulé, C., E. Dargelos, R. Diallo, A. Listrat, D. Béchet, P. Cottin, and S. Poussard. 2010. Proteomic study of calpain interacting proteins during skeletal muscle aging. Biochimie 92:1923–1933.

Carlin, K. R. M., E. Huff-Lonergan, L. J. Rowe, and S. M. Lonergan. 2006. Effect of oxidation, ph, and ionic strength on calpastatin inhibition of μ- and m-calpain. J. Anim. Sci. 84:925–937.

Goll, D. E., V. F. Thompson, H. Q. Li, W. Wei, and J. Y. Cong. 2003. The calpain system. Physiol. Rev. 83:731–801.

Gorg, A., C. Obermaier, G. Boguth, A. Harder, B. Scheibe, R. Wildgruber, and W. Weiss. 2000. The current state of two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis 21:1037–1053.

Huff-Lonergan, E., and S. M. Lonergan. 1999. Postmortem mechanisms of meat tenderization: The roles of the structural proteins and the calpain system. Pages 229–251 in Quality Attributes of Muscle Foods. Y. L. Xiong, C.-T. Ho, and F. Shahidi, ed. Kluwer Academic/Plenum Publishers, New York, NY.

Huff-Lonergan, E., T. Mitsuhashi, D. D. Beekman, F. C. Parrish, D. G. Olson, and R. M. Robson. 1996. Proteolysis of specific muscle structural proteins by μ-calpain at low pH and temperature is similar to degradation in postmortem bovine muscle. J. Anim. Sci. 74:993–1008.

Huff Lonergan, E., W. Zhang, and S. M. Lonergan. 2010. Biochemistry of postmortem muscle—Lessons on mechanisms of meat tenderization. Meat Sci. 86:184–195.

Kim, Y. H., E. Huff-Lonergan, J. G. Sebranek, and S. M. Lonergan. 2010. High-oxygen modified atmosphere packaging system induces lipid and myoglobin oxidation and protein polymerization. Meat Sci. 85:759–767.

Lametsch, R., S. Lonergan, and E. Huff-Lonergan. 2008. Disulfide bond within μ-calpain active site inhibits activity and autolysis. Biochim. Biophys. Acta–Proteins Proteomics 1784:1215–1221.

Lund, M. N., M. Christensen, L. Fregil, M. S. Hviid, and L. H. Skibsted. 2008. Effect of high-oxygen atmosphere packaging on mechanical properties of single muscle fibres from bovine and porcine longissimus dorsi. Eur. Food Res. Technol. 227:1323–1328.

Lund, M. N., M. Heinonen, C. P. Baron, and M. Estevez. 2011. Protein oxidation in muscle foods: A review. Mol. Nutr. Food Res. 55:83–95.

Lund, M. N., R. Lametsch, M. S. Hviid, O. N. Jensen, and L. H. Skibsted. 2007. High-oxygen packaging atmosphere influences protein oxidation and tenderness of porcine longissimus dorsi during chill storage. Meat Sci. 77:295–303.

Maddock, K. R., E. Huff Lonergan, and S. M. Lonergan. 2005. The effect of oxidation on the interaction of calpastatin with μ-calpain. In Int. Congr. Meat Sci. Technol., Baltimore, MD.

Marco-Ramell, A., and A. Bassols. 2010. Enrichment of low-abundance proteins from bovine and porcine serum samples for proteomic studies. Res. Vet. Sci. 89:340–343.

Marouga, R., S. David, and E. Hawkins. 2005. The development of the DIGE system: 2D fluorescence difference gel analysis technology. Anal. Bioanal. Chem. 382:669–678.

Maurides, P. A., G. R. Akkaraju, and R. Jagus. 1989. Evaluation of protein-phosphorylation state by a combination of vertical slab gel isoelectric-focusing and immunoblotting. Anal. Biochem. 183:144–151.

Melody, J. L., S. M. Lonergan, L. J. Rowe, T. W. Huiatt, M. S. Mayes, and E. Huff-Lonergan. 2004. Early postmortem biochemical factors influence tenderness and water-holding capacity of three porcine muscles. J. Anim. Sci. 82:1195–1205.

Mennecke, B. E., A. M. Townsend, D. J. Hayes, and S. M. Lonergan. 2007. A study of the factors that influence consumer attitudes toward beef products using the conjoint market analysis tool. J. Anim. Sci. 85:2639–2659.

Mirza, S. P., and M. Olivier. 2008. Methods and approaches for the comprehensive characterization and quantification of cellular proteomes using mass spectrometry. Physiol. Genomics 33:3–11.

Mullen, A. M., L. Pannier, and R. Hamill. 2009. New insights into the biology of meat quality from genomic and proteomic perspectives, with particular emphasis on beef.

Restuccia, U., E. Boschetti, E. Fasoli, F. Fortis, L. Guerrier, A. Bachi, A. V. Kravchuk, and P. G. Righetti. 2009. Pi-based fractionation of serum proteomes versus anion exchange after enhancement of low-abundance proteins by means of peptide libraries. J. Proteomics 72:1061–1070.

Righetti, P. G., and E. Boschetti. 2010. Blood proteomics and the dynamic range: Some light at the end of the tunnel? J. Proteomics 73:627–628.

Rowe, L. J., K. R. Maddock, S. M. Lonergan, and E. Huff-Lonergan. 2004. Oxidative environments decrease tenderization of beef steaks through inactivation of mu-calpain. J. Anim. Sci. 82:3254–3266.

Sommer, A., and R. R. Traut. 1974. Diagonal polyacrylamide-dodecyl sulfate gel-electrophoresis for identification of ribosomal-proteins crosslinked with methyl-4-mercaptobutyrimidate. Proc. Natl. Acad. Sci. USA 71:3946–3950.

Xiong, Y. L., D. Park, and T. Ooizumi. 2009. Variation in the cross-linking pattern of porcine myofibrillar protein exposed to three oxidative environments. J. Agric. Food Chem. 57:153–159.