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Multi-parameter evaluation of the effect of processing conditions on meat protein modification

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ABSTRACT

Evaluating the interconnecting effects of pH, temperature and time on food proteins is of relevance to food processing, and food functionality. Here we describe a matrix-based approach in which meat proteins were exposed to combinations of these parameters, selected to cover coordinates in a realistic processing space, and analyzed using redox proteomics. Regions within the matrix showing high levels of protein modification were evaluated for oxidative and other modifications. Both pH and temperature, independently, had a significant effect on the oxidative modifications mostly detected in myofibrillar proteins such as myosin and troponin and also collagen. Heat induced pyroglutamic acid formation was exclusively observed in the myofibrillar proteins. Potential interdependencies between pH, temperature and exposure time were evaluated using a 3-way analysis of variance (ANOVA) on protein modification levels to better understand how industry relevant process parameters influence protein quality and function.

1. Introduction

Proteins are an important source of macronutrients and constitute a diverse range of biological macromolecules that influence the functional properties of food. The stability of proteins, determined by their inherent structure and their association with other compounds in food such as fats and sugars, influence the extent to which proteins are modified during food processing (Cheah and Ledward, 1996; Sun-Waterhouse et al., 2014). Different conditions used in food processing can cause proteins to unfold to various degrees which in turn makes them susceptible to aggregation and also prone to chemical modifications such as non-enzymatic glycation, oxidative and heat and pH induced modifications (Ames, 1990; Liao et al., 2010; Lassé et al., 2015).

For foods, particularly in their processed or cooked forms, non-enzymatic glycation or Maillard reaction involves the reaction of reducing sugars with free amino groups of proteins to form Maillard Reaction Products (MRPs). These comprise a family of protein modifications of critical concern to the industry, as they can pose both risks and benefits to the consumer (Dyer et al., 2016). For instance, impaired nutritional value can occur due to changes in protein integrity and function through cross-linking mediated by advanced glycation end products (AGEs) (Friedman, 1996; Matsui et al., 2016). On the positive side, increased antioxidant activity and flavor development in food systems can also be attributed to certain direct and indirect products of these reactions (Bailey and Shahidi, 1994; van Boekel et al., 2010).

Factors such as water activity and concentration of proteins in food can influence their degree of denaturation, aggregation and the extent of modification. Food processing conditions, especially involving heat, can induce chemical changes such as pyrolysis, peptide backbone hydrolysis, formation of adducts with lipids, sugars and polyphenols and deamidation of glutamine and or asparagine residues (Grosvenor et al., 2011; Hu et al., 2017, Tzer-Yang et al., 2017). These reactions can affect the functional properties of food, enhance or reduce nutritional qualities and impart cooking-associated flavors.

Proteins are important not only for their nutritional value, but also as an important contributory factor towards food structure and consumer perception of a food product. Physico-chemical conditions such as variations in pH, temperature, ionic strength and pressure during food processing can strongly influence the functional behavior of proteins (Aryee et al., 2018). Proteins in food can lose their native structure during

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Table 1. Combinations of pH, time and temperature used for treating meat powder samples.

Sample No.	Time (min)	pH	Temp (°C)
1	180	3	60, 70, 80
2	210	3	60, 70, 80
3	240	3	60, 70, 80
4	5	4	60, 70, 80
5	30	4	60, 70, 80
6	60	4	60, 70, 80
7	180	4	60, 70, 80
8	210	4	60, 70, 80
9	240	4	60, 70, 80
10	5	6	60, 70, 80
11	30	6	60, 70, 80
12	60	6	60, 70, 80
13	5	8	60, 70, 80
14	30	8	60, 70, 80
15	60	8	60, 70, 80
16	180	8	60, 70, 80
17	210	8	60, 70, 80
18	240	8	60, 70, 80
19	180	9	60, 70, 80
20	210	9	60, 70, 80
21	240	9	60, 70, 80

processing. Unfolded or hydrolyzed proteins can have very different functional properties to that of native or folded proteins. During the denaturation or unfolding process newly exposed amino acids can undergo modifications to various extents. Variations in reactivity between amino acids present in specific proteins may result due to their differences in exposure to the environment and hence their differences in modification levels (Nguyen et al., 2018). A prevalent modification resulting from food processing is the deamidation of glutamine and asparagine to glutamic and aspartic acid respectively. Low level deamidation has been shown to improve the functional properties such as solubility, emulsifying and foaming properties of gluten (Piovesana et al., 2015). On the other hand, it can also increase hydrophobicity and surface activity of proteins due to induced conformational changes (Cabra et al., 2007). Strong deamidation can influence electrostatic interactions of proteins resulting in protein unfolding due to electrostatic repulsion (Cunsolo et al., 2007).

Although protein functional behavior is not always predictable in nature, it provides an opportune component for manipulations for the best utilization of proteins as nutritive ingredients in food. This is important for food industries as proteins are generally costly and optimized use of protein availability and functionality is always desirable. Industry focus on food quality parameters particularly around nutrition and their impact on consumers form an important part of food processing. Identification of process-induced modifications in food therefore becomes important to understand changes in protein functionality which may result in designing methods to retain protein chemical integrity by preventing unfavorable amino acid modifications. Protein integrity is important for retaining protein digestibility and consequently bioavailability (Schonfeldt et al., 2016).

In this study, we built a data model using bovine muscle to track and predict how changes in industry relevant parameters such as temperature, pH and exposure time affect modification of meat proteins. The model can be visualized as a three-dimensional space (matrix) in which x, y and z coordinates define variation in temperature, pH and time of exposure respectively. Within this space, we concentrated on identifying regions where combinations of these parameters resulted in high levels of amino acid modifications and change was rapid with small changes in parameters ("hot spots"). Models were developed by first defining the entire space in low resolution, and then defining the areas surrounding hot spots in higher resolution to better understand the effect of processing conditions on meat protein quality.

2. Materials and methods

2.1. Materials

Acetonitrile (ACN) and LCMS grade water were obtained from Mallinckrodt (Phillipsburg, NJ, USA). Tris, dithiothreitol (DTT) and formic acid (FA) were from Merck (Darmstadt, Germany). TPCK-trypsin was obtained from Promega Corporation (Madison, WI, USA). Tris(2-carboxyethyl) phosphine (TCEP) was from Fluka Chemie (Buchs, Germany). All other chemicals were of analytical grade.

2.2. Methods

2.2.1. Sample preparation

Fresh bovine meat was obtained from a commercial meat processing facility. 500 g beef muscle (*biceps femoris*) from an animal aged 18 months was measured at pH 5.8 and was stored at 4 °*C prior* to sample preparation. The meat was first sliced into small pieces and then ground with Celite 545 (1:3 ratio) with liquid nitrogen (Leslie Hart and Fisher, 1971). This was then freeze-dried (Dura-Dry μP freeze-dryer, FTS Systems) to obtain a meat powder and stored at -80 °C until further use.

An initial experiment (Experiment 1) was carried out wherein the meat powder samples were exposed to a combination of time, pH and temperature. Samples were exposed to either pH 3, 6 or 10 for 5, 120 or 240 min at temperatures 50 °C, 75 °C or 100 °C. In order to maintain the reaction mixture pH at 3, 6 or 10, 0.1 M citrate, 0.1 M phosphate and 0.1 M tetraborate buffers were used respectively.

To each meat sample (50 mg), 500 μL of a pH adjusted buffer was added and the incubation was performed in a Thermomixer (Eppendorf) with constant shaking. After the incubation step, a methanol/chloroform precipitation (Tidona et al., 2011) was performed on each sample to precipitate soluble proteins onto the insoluble fraction and also to remove buffer salts. The protein pellet obtained from the precipitation step was then dried under vacuum and reconstituted in 50mM ammonium bicarbonate and then reduced with 50 mM tris(2-carboxyethyl) phosphine hydrochloride (TCEP) in 100 mM ammonium bicarbonate at

Table 2. List of heat-induced amino acid modifications with target amino acids.

	Modification	Unimod accession #	Target Amino Acids	Position	Chemical Change
1	Oxidation	35	CMFHPWY	Any	O(1)
2	Dioxidation	425	CMFHPWY	Any	O(2)
3	Trioxidation	345	CFHWY	Any	O(3)
4	Nitration	354	FHWY	Any	H(-1) N(1) O(2)
5	Kynurenine	351	W	Any	C(-1) O(1)
6	Hydroxykynurenine	350	W	Any	C(-1) O(2)
7	Quinone	392	YW	Any	H(-2) O(2)
8	Carbamylation	5	Any	N-terminal	H(1) C(1) N(1) O(1)
9	Deamidation	7	NQ	Any	H(-1) N(-1) O(1)
10	Dehydration	23	S	Any	H(-2) O(-1)
11	Didehydro	401	T	Any	H(-2)
12	Dehydroalanine	400	Y	Any	H(-6) C(-6) O(-1)
13	Dehydroalanine	368	C	Any	H(-2) S(-1)
14	Pyro-glu	27	E	N-terminal	H(-2) O(-1)
15	Pyro-glu	28	Q	N-terminal	H(-3) N(-1)
16	Hex(2)	512	KR	Any	C(12) H(20 O(10)
17	Carboxymethylation	6	K	Any	H(2) C(2) O(2)

56 °C for 45 min, followed by alkylation with 150 mM iodoacetamide in 100 mM ammonium bicarbonate for 30 min at room temperature in the dark. Proteins were digested with sequencing grade trypsin at an enzyme to substrate ratio of 1:60 (w/w) in 50 mM ammonium bicarbonate containing 10% acetonitrile, at 37 °C for 18 h. Sample were then dried using a CentriVap (Labconco, USA) at 30 °C and re-suspended with 0.1% formic acid. The resulting peptides were purified using Empore™ disks (3M) as previously described (Koehn et al., 2011).

In the second experiment (Experiment 2), specific combinations of time, pH and temperature were used to treat the meat powder, as shown in Table 1. Experiment 2 was a 3-way factorial experiment, that examined sub-regions of the sampling space that analysis of Experiment 1 data had identified as having a high level of modification ("hot spots"). Experiment 2 focused on three areas where a high-resolution sampling of the space was performed (thus increasing precision in key areas). These three hot spots were: (1) low pH (pH 3 or 4) for long exposure times (180, 210 or 240 min), (2) medium pH (pH 4, 6 or 8) for short exposure times (5, 30 or 60 min), and (3) high pH (pH 8 or 9) for long exposure times (180, 210 or 240 min). For each hot spot, the third factor, temperature, was set at three levels: 60, 70 or 80 °C. As with the previous experimental conditions, 0.1 M citrate buffer was used for pH 3–4, 0.1 M phosphate buffer for pH 6–8 and 0.1 M tetraborate buffer for pH 9.

2.2.2. Total fat analysis

Approximately 5 g of the raw meat powder was boiled gently for 30 min using 50 μL 6 M HCl. The hydrolysate thus obtained was filtered using a Whatman 54 filter paper and the residue rinsed with water until a neutral pH was reached. The filter paper containing the residue was then dried and Soxhlet extracted to determine the total fat content (Egan et al., 1981). The total fat content was determined to be 2.8 %.

2.2.3. Determination of pH

The pH of the raw meat powder suspension in double distilled water, was measured at ambient temperature using a pH meter (Mettler-Toledo, USA), and was determined to be 5.58.

2.2.4. LC-MS/MS analysis

Nanoflow LC-MS/MS was performed on a nanoAdvance UPLC coupled to an amaZon speed ETD ion trap mass spectrometer equipped with a CaptiveSpray ion source (Bruker Daltonik, Bremen, Germany).

Samples were loaded at 5 μL/min onto on a C18AQ Nanotrap (Bruker, C18AQ, 5 μm, 200 Å) using solvent A (0.1% formic acid), which was then switched in-line with an in-house packed analytical column (100 μm ID x 150 mm) containing Magic C18AQ (3 μm, 200 Å; Bruker). Elution was performed at 800 nL/min, using a linear gradient from 2% to 45% B (98% acetonitrile, 0.1% formic acid) in 60 min. The column oven temperature was maintained at 50 °C. Automated information dependent acquisition (IDA) was performed for each MS spectrum (m/z 350–1200) which was followed by three MS/MS spectra (m/z 40–1600) during each acquisition cycle of 1.3 s duration.

2.2.5. Data analysis

Database search parameters: After each LC-MS/MS run, the peak list data was extracted using DataAnalysis v4.2 (Bruker) followed by protein identification using ProteinScape v3.1 (Bruker). Peak lists were queried against *Bos taurus* sequences in the NCBInr database (release date: 14th September 2011) using the Mascot search engine (v2.2.06, Matrix Science) maintained on an in-house server. The following Mascot search parameters were used: 'semitrypsin' as the proteolytic enzyme with two missed cleavages permitted; fixed modification carbamidomethyl (C); 0.3 Da error tolerance for MS and 0.6 Da for MS/MS. Search results were compiled and analysed using the ProteinExtractor function in ProteinScape. Peptide significance threshold $p < 0.05$ was used. Peptide acceptance threshold of 20 and protein acceptance threshold of 60 with at least one peptide with a score higher than the identity threshold determined by the search engine was required for protein identification and results assessed as true matches were used for further analysis.

Peptide modifications were evaluated using Mascot searches using a combination of up to a maximum of four of the target modifications as variable amino acid modifications at any given time, as shown in Table 2. Error tolerant searches did not produce any additional meaningful combinations of modifications that were not included in the combinations reported.

Redox proteomic analysis: Modifications between samples were compared using an in-house developed scoring system (Dyer et al., 2010; Deb-Choudhury et al., 2014). For the oxidative modifications, first the number of individual oxidatively modified amino acids was obtained. This was then multiplied by weighing factors associated with damage hierarchies that reflected the relative severities of the modifications of the respective unmodified amino acid residue. A total oxidative weighted score was then obtained by summing the individual weighted modification scores as shown in the following equation:

$$S_w = \sum_{i=1}^{n} \left(\frac{aa_{mod_i}}{aa_{tot_i}} \times f_{mod_i} \right)$$

where:

S_w = total weighted modification score.

Table 3. Peptides along with their associated modified amino acids and their proteins of origin, used for the calculation of modification scores.

Sequence	Modifications present on amino acid positions within the peptide sequence	Protein Accession number
LNVKNEELDAMM	Oxidation: 11; Oxidation: 12	gi|115497166
FGEKLKGADPEDVITGAFK	Carbamyl: 1; Oxidation: 1	gi|115497166
FNISNGGPAPEAITDKIFQISK	Deamidated: 5	gi|116004023
ETASVTIVVI	Didehydro: 2; Didehydro: 6	gi|119894624
VDKGVVPLAGTNGETTTQGLDGLSER	Deamidated: 12	gi|156120479
GVVPLAGTNGETTTQGLDGLSER	Deamidated: 9	gi|156120479
YASICQQNGIVPIVEPEILPDGDHDLKR	Deamidated: 8	gi|156120479
AGAAASESLFISNHAY	Deamidated: 13	gi|156120479
YASICQQNGIVPIVEPEILPDGDHDLKR	Deamidated: 6; Deamidated: 7	gi|156120479
KIEPELDGSSPVTSHDSSTNGLINFIK	Deamidated: 20	gi|296477774
FYNELRVAPEEHPTLLTEAPLNPK	Tyr->Dha: 2	gi|296482731
KTWATVTPEVK	Didehydro: 2; Didehydro: 5	gi|297465038
DEDETTALVCDNGSGLVK	Carbamyl: 1	gi|4501881
TIDDLEDELYAQK	Carbamyl: 1	gi|58652133
PELNGK	Deamidated: 4	gi|77404273
VGVNGFGR	Deamidated: 4	gi|77404273
AITIFQERDPANIK	Deamidated: 6	gi|77404273
LTGMAFRVPTPNVSVVDLTCR	Deamidated: 12	gi|77404273
VIPELNGK	Deamidated: 6	gi|77404273
IQLVEEELDR	Deamidated: 2	gi|61888866
AGAAASESLFISNHAY	Deamidated: 13	gi|156120479
QVIGTGSFFPK	Gln->pyro-Glu: 1	gi|164448592
VIPELNGKLTGMAFRVPTPNVSVVDLTCR	Carbamyl: 1	gi|2285903
CDNGSGLVKAGFAGDDAPR	Carbamyl: 1	gi|27819614
QMANSS	Dehydrated: 5	gi|296475256
QASTQQIEELKR	Carbamyl: 1	gi|297486814
QAFTQQIEELKR	Gln->pyro-Glu: 1	gi|41386691
CDNGSGLCKAGFAGDDAPR	Dehydrated: 5	gi|297460766
QKYDITNLR	Gln->pyro-Glu: 1	gi|47824864
MFLSFPTTK	Oxidation: 1	gi|576142
TIDDLEDELYAQK	Carbamyl: 1	gi|58652133
QLEDELVSLQK	Gln->pyro-Glu: 1	gi|61888866
QLEDELVSLQKK	Gln->pyro-Glu: 1	gi|61888866
CSKCLQPLASET	Dehydrated: 2; Dehydrated: 10	gi|77735635
QLEDELVSLQK	Gln->pyro-Glu: 1	gi|61888866
VDKGVVPLAGTNGETTTQGLDGLSER	Deamidated: 12	gi|156120479
ALANSLACQGK	Deamidated: 4	gi|156120479
ACLKKSADTLWGIQK	Oxidation: 2	gi|27806559
SYCMLQGSPLDVLK	Tyr->Dha: 2; Cys->Dha: 3	gi|300796816
HMCRLDIDSPPITAR	Oxidation: 2	gi|73587283
VGVNGFGR	Deamidated: 4	gi|77404273
NGKYDLDFKSPDDPAR	Deamidated: 1	gi|77736349
EIMILKDKL	Glu->pyro-Glu: 1	gi|297461192
VGVNGFGR	Deamidated: 4	gi|77404273
QLLLTADDR	Gln->pyro-Glu: 1	gi|156120479
LFETRIT	Didehydro: 4	gi|567064
RGTASTTK	Didehydro: 3; Didehydro: 6	gi|219521804
LTILSLQQ	Didehydro: 2	gi|297462693
IQLVEEELDR	Deamidated: 2	gi|61888866
IQLVEEELDR	Deamidated: 2	gi|11875203
TIDDLEDELYAQK	Carbamyl: 1	gi|58652133
QLEEEQQALQK	Gln->pyro-Glu: 1	gi|11875203
KLVIIESDLER	Dehydrated: 7	gi|61888866
CSELEEELK	Dehydrated: 2	gi|58652133
KLQEEIQLK	Deamidated: 3	gi|28189827
QLEDELVSLQK	Gln->pyro-Glu: 1	gi|61888866
AEKETELSLQKEQLQLK	Deamidated: 10; Deamidated: 13	gi|296480119

Table 4. Mean total modification score by temperature in three hot spots showing high levels of modifications.

Hot spot with high modification levels	Temperature	Mean total modification ±SEM
pH = 4,6&8 and time = 5,30&60	60	0.578 ± 0.093
	70	1.030 ± 0.148
	80	0.617 ± 0.104
pH = 3&4 and time = 180,210&240	60	0.374 ± 0.085
	70	1.205 ± 0.234
	80	0.711 ± 0.079
pH = 8&9 and time = 180,210&240	60	0.707 ± 0.152
	70	0.822 ± 0.145
	80	0.601 ± 0.064
Average of all three hot spots	60	0.557 ± 0.067
	70	1.020 ± 0.101
	80	0.639 ± 0.052

Table 5. Mean oxidative modification score by temperature in three hot spots showing high levels of modifications.

Hot spots with high modification levels	Temperature	Mean ± SEM
pH = 4,6&8 and time = 5,30&60	60	0.149 ± 0.034
	70	0.212 ± 0.037
	80	0.105 ± 0.041
pH = 3&4 and time = 180,210&240	60	0.083 ± 0.033
	70	0.293 ± 0.037
	80	0.185 ± 0.022
pH = 8&9 and time = 180,210&240	60	0.172 ± 0.099
	70	0.508 ± 0.055
	80	0.397 ± 0.058
Average of all three hot spots	60	0.137 ± 0.032
	70	0.320 ± 0.036
	80	0.212 ± 0.036

Table 6. Mean oxidative modification score by pH in three hot spots showing high levels of modifications.

Hot spots with high modification levels	pH	Mean ± SEM
pH = 4,6&8 and time = 5,30&60	4	0.115 ± 0.044
	6	0.170 ± 0.037
	8	0.181 ± 0.036
pH = 3&4 and time = 180,210&240	3	0.219 ± 0.032
	4	0.155 ± 0.042
pH = 8&9 and time = 180,210&240	8	0.274 ± 0.070
	9	0.444 ± 0.070
Average of all three regions	3	0.219 ± 0.032
	4	0.135 ± 0.030
	6	0.170 ± 0.037
	8	0.227 ± 0.040
	9	0.444 ± 0.070

aa_{mod_i} = count of amino acids affected by the i^{th} modification.

aa_{tot_i} = total number of amino acids that could be affected by the i^{th} modification.

f_{mod_i} = the weighing factor for the i^{th} modification.

A similar approach was used for calculating the total scores for Maillard and other heat-induced modifications, but without any weighting factors.

Modifications 1–7 were categorized as oxidative, 8–15 as modifications classed as other-than-oxidative in nature and 16–17 as Maillard modifications (Table 2). A list of peptides along with their associated modified amino acids and their proteins of origin is provided in Table 3.

2.2.6. Statistical analysis

To examine the effect of each of the three factors in Experiment 1, a 3-way analysis of variance (ANOVA) was performed separately for the oxidative modification score, other modification score that included Maillard modifications and the total modification score, which was the sum of the oxidative and other modification scores. Since Experiment 1 was a single replicate experiment (factorial experiment with one observation per cell), each ANOVA used the 3-factor interaction (pH*Time*Temperature) as the residual term to allow all 2-factor interaction effects to be examined from the experiment. For single replicate experiments, higher-order factor interactions are used as the residual term,

Table 7. Mean modification score other than oxidative by time and temperature in three hot spots showing high levels of modifications.

Hot spots with high modification levels	Time	Temperature	Mean ± SEM
pH = 4,6&8 and time = 5,30&60	5	60	0.658 ± 0.103
		70	0.871 ± 0.044
		80	0.627 ± 0.100
	30	60	0.249 ± 0.056
		70	1.180 ± 0.308
		80	0.564 ± 0.130
	60	60	0.380 ± 0.189
		70	0.403 ± 0.164
		80	0.345 ± 0.147
pH = 3&4 and time = 180,210&240	180	60	0.498 ± 0.132
		70	0.602 ± 0.411
		80	0.378 ± 0.213
	210	60	0.228 ± 0.070
		70	0.796 ± 0.242
		80	0.570 ± 0.022
	240	60	0.148 ± 0.066
		70	1.337 ± 0.555
		80	0.628 ± 0.106
pH = 8&9 and time = 180,210&240	180	60	0.720 ± 0.280
		70	0.327 ± 0.195
		80	0.099 ± 0.032
	210	60	0.432 ± 0.099
		70	0.111 ± 0.052
		80	0.323 ± 0.236
	240	60	0.452 ± 0.048
		70	0.505 ± 0.273
		80	0.188 ± 0.121
Average of all three regions	5	60	0.658 ± 0.103
		70	0.871 ± 0.044
		80	0.627 ± 0.100
	30	60	0.249 ± 0.056
		70	1.180 ± 0.308
		80	0.564 ± 0.130
	60	60	0.380 ± 0.189
		70	0.403 ± 0.164
		80	0.345 ± 0.147
	180	60	0.609 ± 0.142
		70	0.465 ± 0.202
		80	0.238 ± 0.119
	210	60	0.330 ± 0.077
		70	0.453 ± 0.222
		80	0.446 ± 0.120
	240	60	0.300 ± 0.094
		70	0.921 ± 0.349
		80	0.408 ± 0.143

because these interactions are smaller than main effects and lower-order interactions (Dean and Voss 1999).

A response surface analysis was also performed for each of the three modification scores separately. The purpose of this analysis was twofold:

1) to identify conditions for lesser modification, and
2) To identify regions of the sampling space where the modifications were determined to be high (hot spots).

Experiment 2 was also a single replicate 3-way factorial experiment. Therefore, each score was analyzed in a 3-way ANOVA, by using the 3-factor interaction (pH*Time*Temperature) as the residual term.

3. Results

Meat powder was used as the starting material to expose a larger surface area to insult as rapidly as possible without forming a gradient from the surface to the core, as would be expected if larger meat pieces were used. Individual samples were subsequently investigated for protein modification.

3.1. Experiment 1

Analyses were performed to investigate the effects of three factors; Time, pH and Temperature on the amino acid modifications of meat proteins across ranges which encompass most conditions that may occur during meat processing. Samples were exposed to either pH 3, 6 or 10 for

Table 8. Mean modification score other than oxidative by pH and temperature in three hot spots showing high levels of modifications.

Hot spots with high modification levels	pH	Temperature	Mean ± SEM
pH = 4,6&8 and time = 5,30&60	4	60	0.372 ± 0.061
		70	0.786 ± 0.087
		80	0.493 ± 0.125
	6	60	0.513 ± 0.183
		70	0.718 ± 0.270
		80	0.461 ± 0.203
	8	60	0.403 ± 0.224
		70	0.949 ± 0.427
		80	0.582 ± 0.100
pH = 3&4 and time = 180,210&240	3	60	0.246 ± 0.062
		70	1.041 ± 0.491
		80	0.426 ± 0.132
	4	60	0.336 ± 0.159
		70	0.783 ± 0.133
		80	0.624 ± 0.056
pH = 8&9 and time = 180,210&240	8	60	0.392 ± 0.031
		70	0.141 ± 0.050
		80	0.252 ± 0.154
	9	60	0.677 ± 0.162
		70	0.487 ± 0.178
		80	0.154 ± 0.078
Average of all three regions	3	60	0.246 ± 0.062
		70	1.041 ± 0.491
		80	0.426 ± 0.132
	4	60	0.354 ± 0.077
		70	0.785 ± 0.071
		80	0.559 ± 0.068
	6	60	0.513 ± 0.183
		70	0.718 ± 0.270
		80	0.461 ± 0.203
	8	60	0.398 ± 0.101
		70	0.545 ± 0.264
		80	0.417 ± 0.110
	9	60	0.677 ± 0.162
		70	0.487 ± 0.178
		80	0.154 ± 0.078

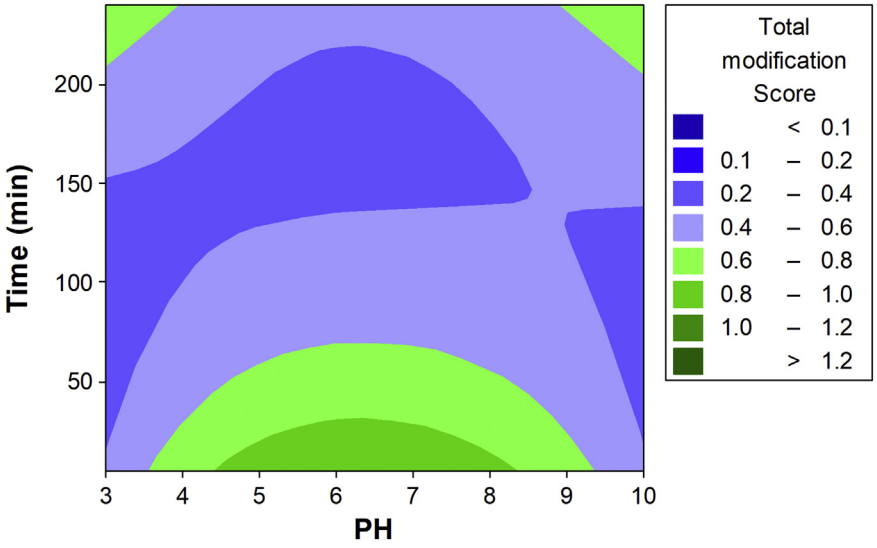

Figure 1. Contour plot of total modification score showing statistically weak interaction (p-value = 0.062), significant at 10% significance level) between Time and pH factors.

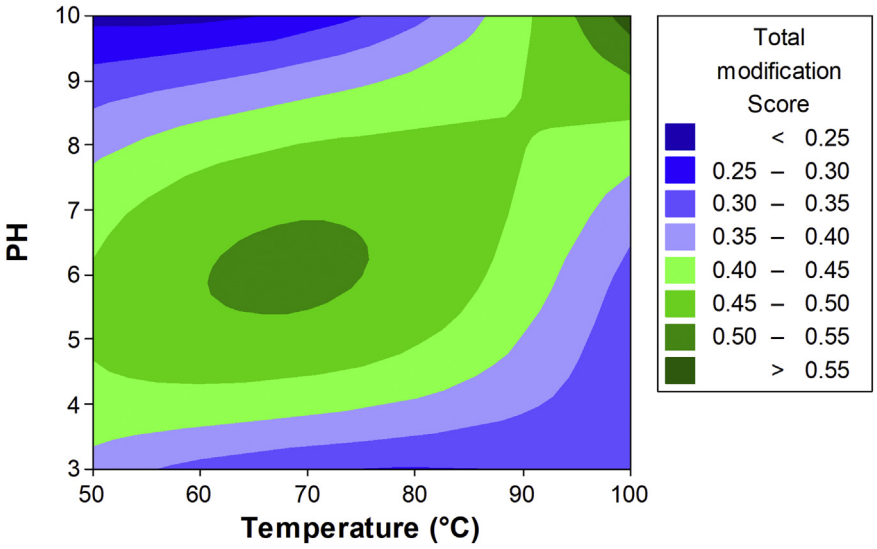

Figure 2. Contour plot of total modification score at Time = 120 min.

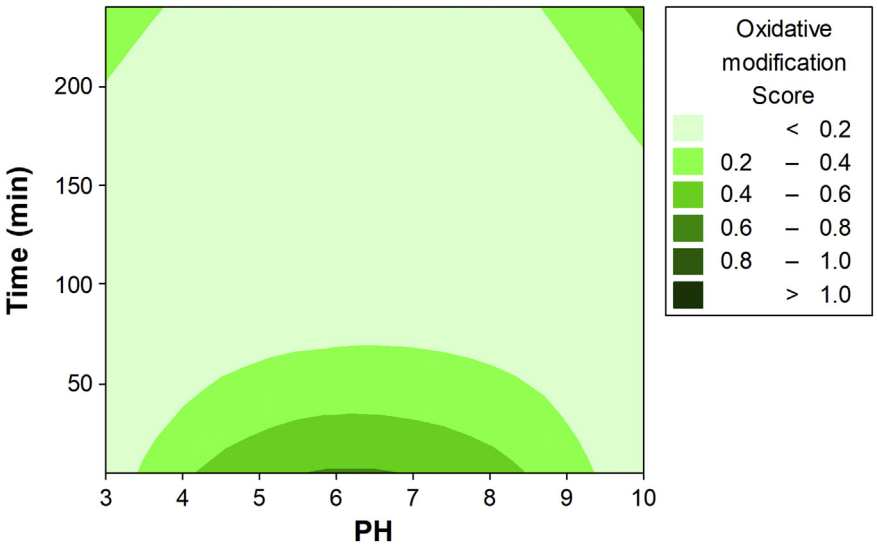

Figure 3. Contour plot of oxidative modification score showing statistically significant interaction (p-value = 0.005) between Time and pH factors.

5, 120 or 240 min at temperatures 50 °C, 75 °C or 100 °C, as a 3-way factorial experiment.

3.1.1. Total modification score

Among all the 2-factor interaction effects, only the Time*pH interaction was found to be significant at the 10% level (p-value 0.062) on the total modification score (Figure 1). In other words, there was weak statistical evidence suggesting that the relationship between pH and total modification score differed depending on exposure time. From the response surface plot, total modification score was found to be low around the time of 120 min. Closer examination at this time of exposure revealed that the total modification score was the lowest at two extreme combinations of pH and temperature: pH 3 at the temperature of 100 °C, and pH 10 at the temperature of 50 °C (Figure 2).

Analysis of the combined effects of all three parameters (response surface plotting) identified three hot spots where the total modification score was high: longer exposure times (180–240 min) with low pH (pH 3–4) or high pH (pH 8–10), and shorter exposure times (5–60 min) with medium pH (pH 4–8) (Figure 1). Of all these hot spots, the highest modifications were made within a medium temperature range of 60–80 °C (Figure 2). Interaction of time and temperature on the total modification score was found to be not significant.

3.1.2. Oxidative modification score

Regarding oxidative modification score, Time*pH (Figure 3) and Time*Temperature (Figure 4) interactions were statistically significant (p-value of 0.005 and 0.025 respectively), indicating that the effects of both pH and temperature on this score are dependent on time. From the response surface plot, the level of oxidative modification was found to be low when the time of sample exposure was 120 min, provided that pH was maintained below 6 and/or temperature was kept below 75 °C.

3.1.3. Other modification score (for modifications categorized as other-than-oxidative)

The other modification score was statistically significant at the 5% level (p-value = 0.017), for the Time*Temperature interactions (Figure 5). This indicated that the effects of temperature on this score were dependent on time. The interaction between pH and Temperature was less significant but close to the 10% level (p-value = 0.102) (Figure 6). The response surface plot predicts that these modifications

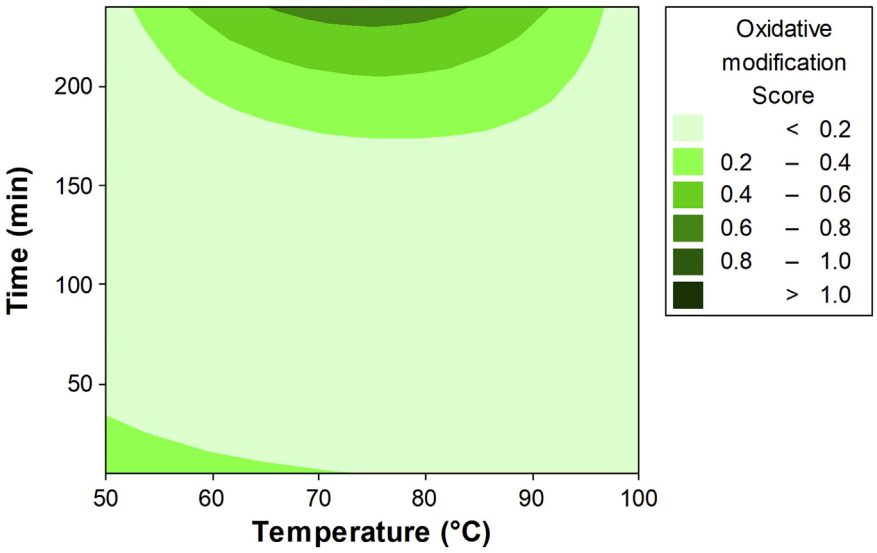

Figure 4. Contour plot of oxidative modification score showing statistically significant interaction (p-value = 0.025) between Time and Temperature factors.

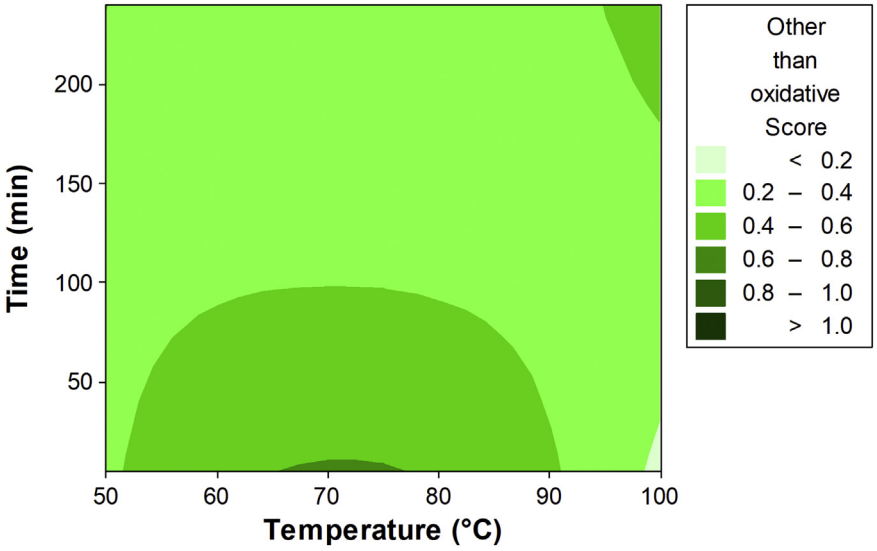

Figure 5. Contour plot of modification other-than-oxidative score showing statistically significant interaction (p-value = 0.017) between Time and Temperature factors.

will be lower at conditions around an exposure time of 5 min and temperature of 100 °C, regardless of pH values.

3.2. Experiment 2

The space surrounding each of the three hot spots identified in Experiment 1 as having statistically significant high modification scores were further analyzed with a larger combination of pH, time and temperature parameters to obtain a greater resolution of the combinatorial effect of these parameters within the space surrounding those hot spots.

3.2.1. Total modification score

Similarly, to Experiment 1, no statistically significant factor interaction was found on total modification score within the three hot spots. Therefore, further ANOVA without interaction terms (Time + pH + Temperature only) was performed to determine each factor's independent but additive effects. From this ANOVA, the effect of temperature on the total modification score was found to be statistically significant (p-value < 0.001). However, the effects of Time and pH factors were not statistically significant within the hotspots.

Since no specific dependencies between Temperature with Time and pH factors were observed, total modification score within each hot spot by Temperature was analyzed using pair-wise comparison between temperatures 60 °C, 70 °C and 80 °C across all Time and pH factor levels (Figure 7). In Figure 7 each symbol in panels A–C represents mean score within each hotspot at the given temperature (averaged across pH and Time values within the hotspot), while each symbol in panel D represents overall mean score across the three hotspots at the given temperature.

These comparisons found that the mean total modification score at 70 °C was statistically significantly larger than those at 60 °C (p-value < 0.001) and 80 °C (p-value = 0.002) across the three hot spots. All other differences were not significant.

3.2.2. Oxidative modification score

As with total modification score, there was no evidence of statistically significant factor dependencies observed within the hotspots for

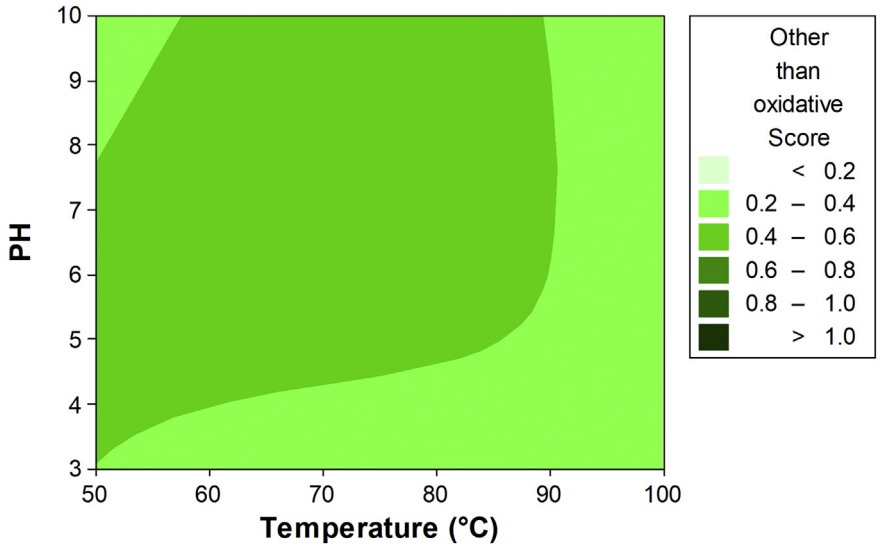

Figure 6. Contour plot of modification other-than-oxidative score showing non-significant pH and Temperature interaction (p-value = 0.102)).

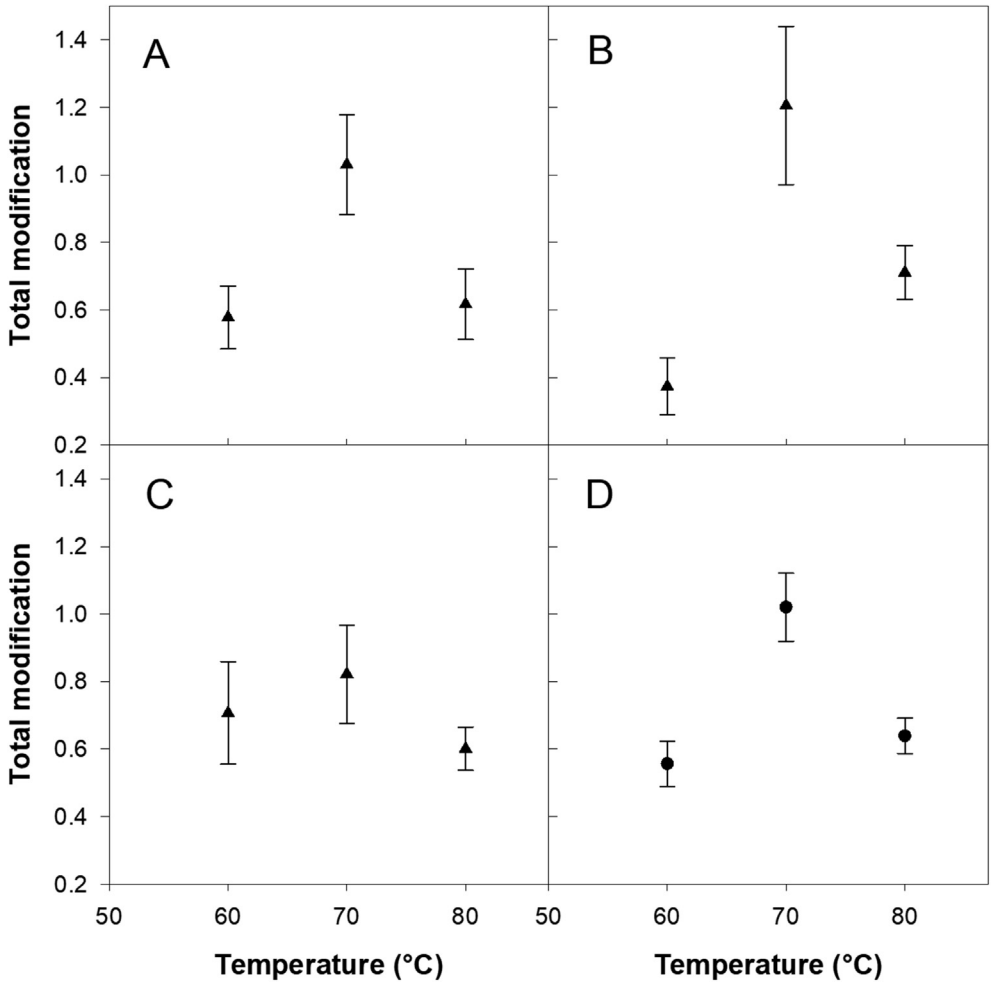

Figure 7. Mean total modification score by temperature in three hot spots showing high levels of modifications – A is pH = 4, 6 & 8 and time = 5, 30 & 60 min, B is pH = 3 &4 and time = 180, 210 & 240 min, C is pH = 8 & 9 and time = 180, 210 & 240 min, and D is average of all three hot spots (data provided in Table 4), with significantly higher scores at 70 °C than at 60 °C and 80 °C (P < 0.001 and P = 0.002, respectively).

oxidative modification score. The ANOVA without interaction terms found statistically significant effects of pH (p-value < 0.001) and temperature (p-value < 0.001), but not of time (p-value = 0.769).

The oxidative modification score in each hot spot by temperature (Figure 8) was compared across the three hot spots, in pair-wise fashion between temperatures 60 °C, 70 °C and 80 °C. These comparisons found

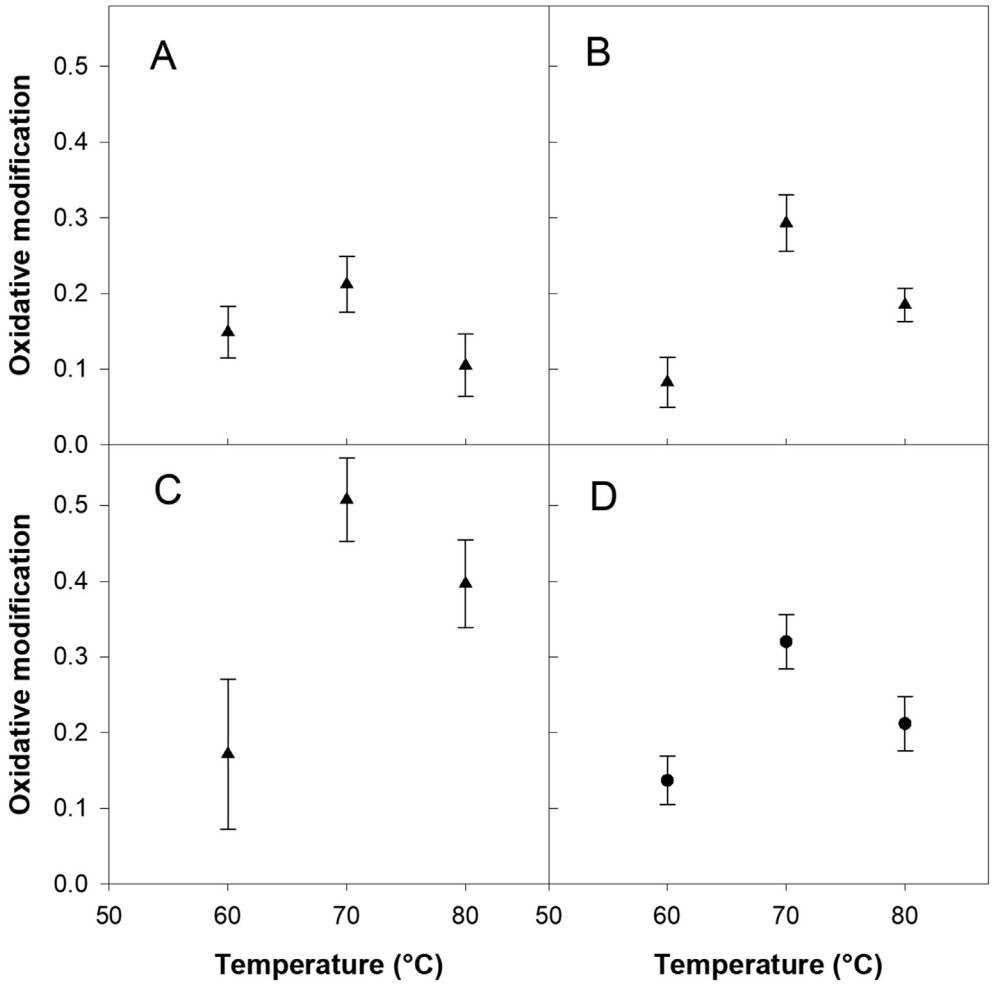

Figure 8. Mean oxidative modification score by temperature in three hot spots showing high levels of modifications – A is pH = 4, 6 & 8 and time = 5, 30 & 60 min, B is pH = 3 &4 and time = 180, 210 & 240 min, C is pH = 8 & 9 and time = 180, 210 & 240 min, and D is average of all three hot spots (data provided in Table 5), with significantly higher scores at 70 °C than at 60 °C and 80 °C ($P < 0.001$ and $P = 0.028$, respectively).

that the mean oxidative modification score at 70 °C was statistically significantly larger than those at 60 °C (p-value < 0.001) and 80 °C (p-value = 0.028) across the hot spots. Similarly, the oxidative modification score by pH level (Figure 9) was next compared in pair-wise fashion between pH values, but only across hot spots with same exposure times. These comparisons found that the mean oxidative modification score at pH 9 was statistically significantly larger when compared with pH 3 (p-value = 0.006), pH 4 (p-value < 0.001) and pH 8 (p-value = 0.021). The comparison between pH 9 and pH 6 was not performed since these two pH levels did not share the same exposure time. All other pair-wise differences were not significant.

3.2.3. Other modification score

Of the modification score for modification classified as other-than-oxidative, Time*Temperature and pH*Temperature interactions were statistically significant at the 10% level (p-values 0.091 and 0.057 respectively, both <0.10). This means that temperature influences this modification score; depending on not only the exposure time but also pH levels.

The Time*Temperature interaction effect on the other-than-oxidative modification score (Figure 10) was examined using pair-wise comparisons. These comparisons found that the mean modification score other-than-oxidative, with Time = 30 min or 240 min at 70 °C, was significantly larger compared to 60 °C or at 80 °C (p-value <0.001, 0.017, 0.019 and 0.049, respectively). Similarly, the pH*Temperature interaction effect on the other than modification score other-than-oxidative (Figure 11) was examined using pair-wise comparisons. These comparisons found that the mean modification score other-than-oxidative, at pH = 3 was significantly larger at 70 °C than at 60 °C or 80 °C (p-value = 0.006 and 0.029, respectively). All other pair-wise differences were not significant.

4. Discussion

This study was conducted to begin to understand and evaluate the combinatorial effects of industrially relevant parameters such as pH, time and temperature on modifications of soluble meat proteins. The formation of modifications at the protein level due to the combinatorial effect of pH, time and temperature is further complicated by the interdependence of the various modification pathways (Deb-Choudhury et al., 2014). The effect of these modifications on the bioavailability and the nutritional quality of proteins in food substrates therefore requires further investigations.

4.1. Processing space (matrix) as a useful approach to encompass processing conditions

The first phase of our experimental approach was designed to monitor the changes in modification levels using a matrix approach wherein specific and extreme combinations of pH, time and temperature were used. This defined a processing space, which might, in practice encompass conditions possible within a processing chamber. Regions within

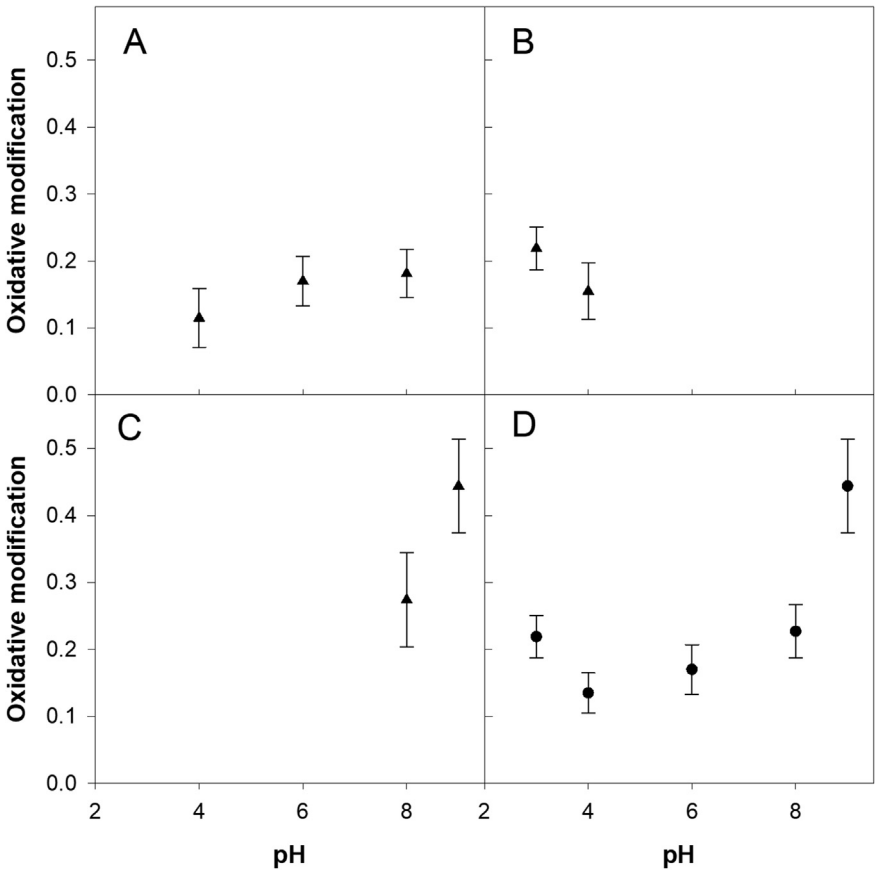

Figure 9. Mean oxidative modification score by pH in three hot spots showing high levels of modifications – A is pH = 4, 6 & 8 and time = 5, 30 & 60 min, B is pH = 3 &4 and time = 180, 210 & 240 min, C is pH = 8 & 9 and time = 180, 210 & 240 min, and D is average of all three hot spots (data provided in Table 6), with significantly higher scores at pH 9 than at pH 3, 4 and 8 ($P = 0.006$, $P < 0.001$ and $P = 0.021$, respectively) (no comparison to pH 6 since pH 6 did not have the same exposure time as pH 9).

this matrix were also seen with varying levels of modifications. The idea behind this approach was to determine conditions under which meat proteins are least and most modified and later extrapolate this to industrial processing conditions for refinement and optimization. Modifications using this matrix approach were divided into three categories, namely oxidative modifications, modifications that are not classified as oxidative (other modifications) and combined total modifications. Scores were assigned to each type as an indicator of their level or severity.

The total modification score was found to be dependent on the combinatorial effect of pH and time. Combination of factors such as temperature and time did not seem to have an effect that was statistically significant. Low total modification values were obtained where the conditions were either maintained at pH 10 and temperature at 50 °C or pH 3 and temperature 100 °C. At 50 °C or pH 10, the common muscle proteins and enzymes such as myosin, aldolase and glyceraldehyde-3-phosphate dehydrogenase contributed most towards the modification score.

4.2. Food chemistry basis of modifications observed in this study

Oxidative modifications were present in myosin and deamidation modification in the enzymes. Methionine and aromatic amino acids such as phenylalanine were oxidatively modified. Previous studies have shown that aromatic amino acids are particularly prone to oxidative modifications (Gatellier et al., 2009a; Gatellier et al., 2009b). Deamidation in food proteins has been reported earlier to be influenced by both temperature and pH (Lassé et al., 2015). Elevated temperatures enhance penetration of water into proteins resulting in protein unfolding and exposing sites previously unavailable for deamidation. Deamidation due to this change in protein conformation results in negatively charged acids that further compound the unfolding process exposing more internal amides (Grosvenor et al., 2011; Thomas et al., 2017).

The total modification score of the sample exposed at the higher temperature of 100 °C or pH 3 was largely derived from high levels of deamidation and the formation of pyroglutamic acid. Previous studies reported that a high percentage of deamidation in proteins occurs at an acidic pH due to the direct hydrolysis of the amide bond (Thomas et al., 2017). Formation of pyroglutamic acid was observed in proteins such as myosin, troponin and tropomyosin. This is a heat-induced modification that is not related to glycation or oxidation of proteins but results from a cyclisation reaction in which the free amino group of glutamine or glutamic acid forms a cyclic amide, lactam. This type of modification has been earlier reported in milk and meat proteins exposed to heat (Meltretter and Pischetsrieder, 2008; Deb-Choudhury et al., 2014).

To better understand how oxidative modifications and other modifications contributed towards this total modification score, they were analyzed separately. The oxidative modification score was found to be dependent on the combinatorial effects of time with either pH or temperature, but not pH and temperature. Oxidative modification was not observed on prolonged exposure of samples to 75 °C at an acidic pH. It is possible that this is due to severe protein aggregation under these conditions. Aggregated proteins are difficult to digest using trypsin for subsequent proteomic analysis and therefore these results could be due to sub-optimal trypsin digestion of proteins producing fewer tryptic peptides for further proteomic analysis. Protein aggregates can vary in size and morphology and are influenced by the net charge in the proteins (Roberts, 2014). Protein aggregation plays a major role in food science and has been shown to take place as a result of processing conditions (Promeyrat et al., 2010; Deb-Choudhury et al., 2014).

The combinatorial effect of time and temperature was statistically significant for modifications which were not specifically classed as oxidative. These modifications were mostly deamidation and the formation of pyroglutamic acid. The level of deamidation was comparatively high, indicating proteins undergo this type of modification quite

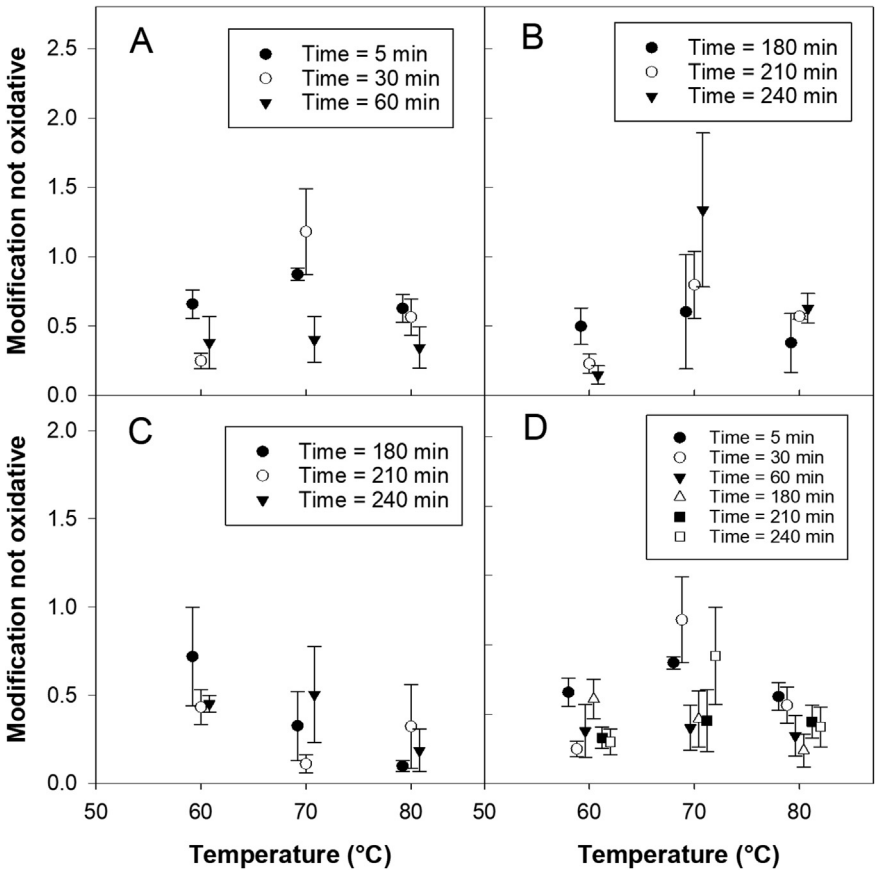

Figure 10. Mean modification score other-than-oxidative by time and temperature in three hot spots showing high levels of modifications – A is pH = 4, 6 & 8 and time = 5, 30 & 60 min, B is pH = 3 &4 and time = 180, 210 & 240 min, C is pH = 8 & 9 and time = 180, 210 & 240 min, and D is average of all three hot spots (data provided in Table 7), with significantly higher scores at 70 °C than at 60 °C and 80 °C when time = 30 min ($P < 0.001$ and $P = 0.017$, respectively) and time = 240 min ($P = 0.019$ and 0.049, respectively).

easily. Deamidation can induce protein conformational change such as protein unfolding due to electrostatic repulsion (Thomas et al., 2017). which can lead to further modifications of amino acids.

4.3. Advantages and limitations of the matrix analysis approach

While this matrix approach works well for establishing where to look for more extreme effects of processing, the challenge is selecting a resolution that is useful to the specific process being studied, as each point requires significant resource to obtain modification data (even more so when increasing numbers of parameters are included). The two-stage approach we took is a good compromise between high resolution and realistic practicality, in which the region of interest (high modification rates) was first identified from a low-resolution matrix (Experiment 1), then further investigated in greater detail at high resolution (Experiment 2). With such multi-stage approaches a larger number of processing variables can be investigated to gain a better understanding of how the variables could result in discernable changes in the modification levels, on their own or as combinations. Focusing on hotspots is particularly important if specific regions of parameter space are more sensitive to changes than other regions.

4.4. Use of "hot spots" to help identify how combined factors affect complex meat proteins

In our study the total modification scores analyzed within these 'hot spots' did not reveal statistically significant dependencies between pH, time and temperature factors. In other words, we observed that each of these factors influenced the total modification score independent of each other. Among these, temperature was found to have a significant effect on the level of modification.

On comparison of the total modification scores at three different temperature levels, it was found that soluble sarcoplasmic meat proteins are vulnerable to a high degree of modifications as the temperature reaches 70 °C. This is due to their easy extractability from the meat protein matrix. The sarcoplasmic proteins are a mixture of several hundred molecular species and several of these are enzymes of the glycolytic pathway (Marino et al., 2014). Sarcoplasmic proteins/enzymes such as aldolase, creatin kinase, phosphorylase, myoglobin, enolase, anhydrase and glyceraldehyde phosphate dehydrogenase contributed majorly towards the overall total modification score. Myofibrillar proteins and connective tissue proteins however contributed less towards the overall score. Only some instances of modifications in tropomyosin, troponin and collagen were seen at this temperature. These modified proteins were also seen in the samples that were exposed at 60 °C and 80 °C albeit with a lower contribution to the overall score in comparison to the 70 °C exposed sample.

Oxidative modifications and modifications other-than-oxidative were also analyzed individually in these hot spots. No statistically significant factor interactions between time, temperature and pH influenced the oxidative scores. However, pH or temperature, independently had a significant effect on the oxidative scores. Oxidative modifications significantly increased between 60 °C and 80 °C and were detected only in myofibrillar proteins such as myosin and troponin and in collagen. The most prominent modification that was not oxidative in nature was deamidation. Deamidation was well distributed in both sarcoplasmic as well as myofibrillar proteins. Heat induced pyroglutamic acid formation was exclusively present in the myofibrillar proteins.

Samples exposed to 80 °C exhibited an expected increase in the levels of deamidation compared to the 70 °C samples. It was also noted that samples exposed to pH 6 and above were modified more than samples below pH 6. Higher modifications observed in samples exposed to pH 6 and above could be due a shift away from the isoelectric point of

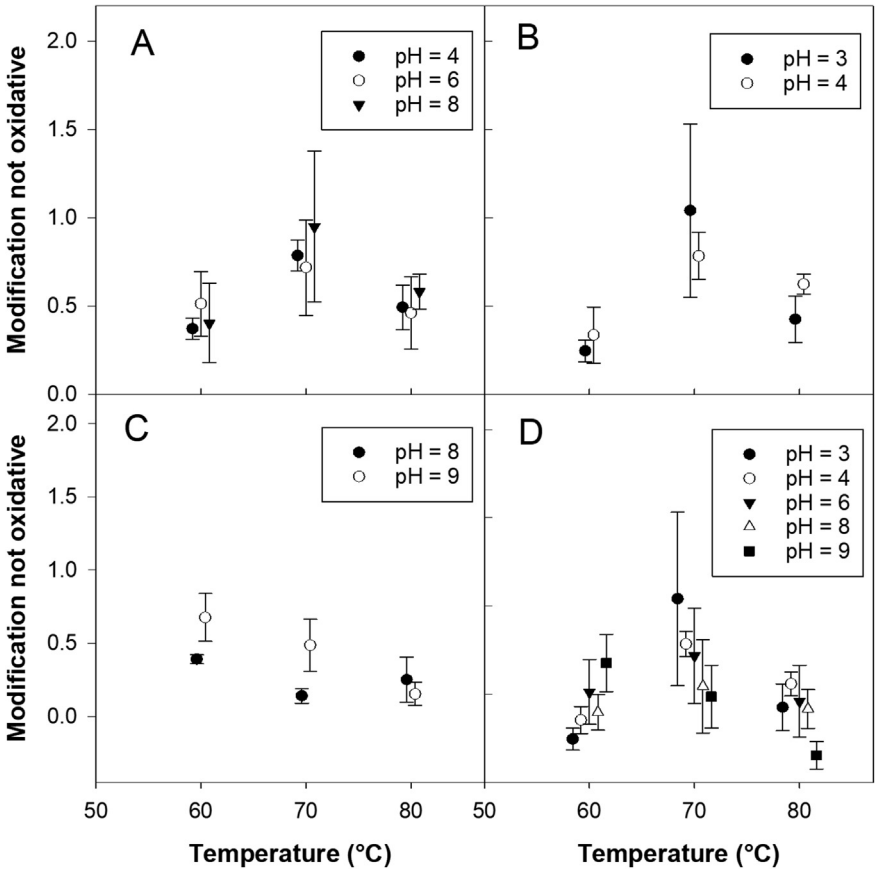

Figure 11. Mean modification score other-than-oxidative by pH and temperature in three hot spots showing high levels of modifications – A is pH = 4, 6 & 8 and time = 5, 30 & 60 min, B is pH = 3 &4 and time = 180, 210 & 240 min, C is pH = 8 & 9 and time = 180, 210 & 240 min, and D is average of all three hot spots (data provided in Table 8), with significantly higher scores at 70 °C than at 60 °C and 80 °C at pH 3 (P = 0.006 and 0.029, respectively).

myofibrillar proteins resulting in a larger number of peptides that could be analyzed after the trypsin digestion phase. The isoelectric point of myofibrillar proteins is near pH 5. At a pH below 6 myofibrillar proteins will tend to aggregate resulting in sub-optimal trypsin digestion. Proteins aggregate near their isoelectric point due to reduced electrostatic repulsion and exposure of hydrophobic moieties (Ju and Kilara, 1998).

5. Conclusions

Protein substrates are complex and react heterogeneously across multi-dimensional space defined by processing parameter continua. Our "matrix" approach allowed us to identify regions of interest within processing space ("hot spots") for examination in more detail. Overall the results provided a better understanding of industry relevant process parameters such as pH, temperature and time of exposure on protein modifications. Monitoring protein modifications in the food matrix is important as it provides a link between processing space and the effect these modifications may have on protein functionality and consequently their bioavailability.

Declarations

Author contribution statement

Santanu Deb-Choudhury, Stephen Haines, Duane Harland: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Stefan Clerens: Conceived and designed the experiments; Wrote the paper.

Chikako van Koten, Jolon Dyer: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Erin Lee, Ancy Thomas: Performed the experiments; Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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