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OXIDAÇÃO LIPÍDICA E PROTEICA DE UM PRODUTO
EMULSIONADO TIPO HAMBÚRGUER DE FRANGO

JOÃO PESSOA

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Tese apresentada ao Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, Centro de Tecnologia, Universidade Federal da Paraíba, em cumprimento aos requisitos para obtenção do título de doutor.

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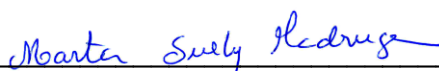
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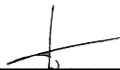
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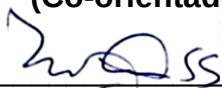
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A minha mãe, meu pai e minha irmã – minhas riquezas,
Ao meu amado esposo e nossa filha Júlia,
Os meus maiores incentivos e incentivadores,
Dedico.

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RESUMO

Alimentos prontos para consumo, como o hambúrguer, estão sendo cada vez mais apreciados pelos consumidores em virtude da demanda atual de uma população que desprende cada vez menos tempo para o preparo de alimentos. Porém, durante as etapas de processamento destes alimentos, a moagem, moldagem, pré-cozimento, embalagem, armazenamento e o reaquecimento antes do seu consumo podem impulsionar sua deterioração oxidativa. Com o intuito de minimizar os efeitos negativos das oxidações lipídica e proteica na qualidade de produtos cárneos, a indústria faz uso de diferentes estratégias, dentre as quais se destacam o uso de antioxidantes e embalagens com atmosfera modificada (MAP). Neste contexto, objetivou-se avaliar o impacto que diferentes tipos de cozimento (imersão em água quente, forno e chapa), tipo de embalagem (ar sintético e MAP), uso de antioxidante natural (extrato de *Quercus ilex*) e reaquecimento em micro-ondas na deterioração físico-química, valor nutritivo, aceitação sensorial, digestibilidade *in vitro* e oxidação de lipídios e proteínas em emulsionados tipo hambúrguer de frango prontos para consumo, armazenados sob refrigeração (+4°C) por um período de 14 dias. A caracterização dos emulsionados tipo hambúrguer foi realizada a partir das determinações da composição química (umidade, proteínas e lipídios), ácidos graxos, parâmetros de cor (L^* , a^* e b^*), dureza, e perda de peso por cocção. O impacto dos fatores citados no desenvolvimento da oxidação lipídica foi medido a partir das análises de TBARS. Para a oxidação das proteínas foram realizadas análises de compostos carbonílicos, triptofano, bases de Schiff, tióis livres e ligações dissulfeto. Os compostos voláteis, compostos de Maillard, digestibilidade *in vitro* e aceitação sensorial (cor e odor) também foram determinados. Os tipos de cozimento aplicados influenciaram a composição química, a deterioração do odor e o desenvolvimento da oxidação lipídica dos emulsionados de frango prontos para consumo. O cozimento em água quente provocou maior oxidação nas proteínas em comparação com os demais métodos, entretanto durante o período de armazenamento muitas modificações consideráveis aconteceram nos cozimentos em forno e em chapa. A oxidação das proteínas parece estar relacionada com as alterações de textura e digestibilidade *in vitro* dos emulsionados tipo hambúrguer. O reaquecimento em micro-ondas teve menos impacto no desenvolvimento oxidativo quando comparado com o tempo de armazenamento dos emulsionados tipo hambúrguer. A adição do extrato vegetal foi uma estratégia efetiva na inibição do início dos processos oxidativos lipídico e proteico, e preservou a qualidade dos emulsionados tipo hambúrguer reaquecidos, podendo ser comparados aos recém cozidos. A aplicação de extrato vegetal, com potencial antioxidante, foi mais eficiente no controle das oxidações lipídica e proteica do que o uso de embalagem com baixa concentração de oxigênio. Assim, com os resultados gerados nesta pesquisa, foi possível concluir que a aplicação de diferentes tipos de cozimento, o uso de embalagem com atmosfera modificada e o uso de extrato vegetal apresentam efeito sobre a estabilidade oxidativa, as características de qualidade e aceitação sensorial do odor dos emulsionados tipo hambúrguer de frango.

Palavras chave: oxidação lipídica e proteica, alimentos prontos para consumo, embalagem com atmosfera modificada, antioxidante natural.

ABSTRACT

Ready-to-eat foods has been increasingly appreciated by consumers due to the current demand of a population that have less time to prepare food. However, during the processing steps of ready-to-eat foods, grinding, molding, pre-cooking, packaging, storage and reheating before consumption may accelerate the oxidative deterioration of the product. Aiming to minimize the negative effects of lipid and protein oxidation on meat products quality, meat industry has used different strategies such as antioxidants and modified atmosphere packaging (MAP). In this sense, the aim of this study was to assess the impact of different cooking methods (boiling, roasting and grilling), packaging types (synthetic air and MAP), natural antioxidant and microwave reheating on the physicochemical deterioration, nutritional value, sensory acceptance, *in vitro* digestibility and on lipid and protein oxidation of ready-to-eat chicken patties stored at 4°C during 14 days. Regarding to ready-to-eat chicken patties characterization, chemical composition (moisture, protein, lipid), fatty acids, instrumental color (L^* , a^* and b^*), hardness and cooking loss were performed. The impact of the factors on the development of lipid oxidation in ready-to-eat patties was measured by TBARS assessment. Carbonyl compounds, tryptophan fluorescence, Schiff Bases, free thiols, disulfide bonds and *in vitro* digestibility were assessed to evaluate protein oxidation. Volatile compounds, Maillard products, *in vitro* digestibility and sensory acceptance (color and odor) were also determined. The cooking methods influenced chemical composition, odor deterioration and the evolution of lipid oxidation in ready-to-eat chicken patties. Boiled samples presented high protein oxidation compared to other ones. However, during the storage, noticeable changes occurred in roasted and grilled samples. Protein oxidation in ready-to-eat chicken patties seems to be related to changes in texture and *in vitro* digestibility. The microwave reheating had minor influence on oxidative deterioration of ready-to-eat samples compared to storage time. The addition of oak nut extract was effective on the onset of lipid and protein oxidation, preserving the quality of ready-to-eat chicken patties after microwave reheating. In our experiment, the use of plant extract with antioxidant potential in ready-to-eat chicken patties processing was more efficient than low oxygen packaging to control of lipid and protein oxidation. In conclusion, it was possible observe that the application of different cooking methods, the use of MAP and addition of oak nut extract in ready-to-eat chicken patties have effect on its stability in terms of oxidative deterioration and sensory acceptance.

Keywords: Lipid and protein oxidation, ready-to-eat foods, Modified Atmosphere Packaging, natural antioxidant.

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LISTA DE ABREVIATURAS E SIGLAS

AAA - Ácido α -aminoadípico
AAS - Semialdeído α -aminoadípico
AU - Unidades de área
BHA - Terc-butil-hidroxianisol
BHT - Hidroxitolueno butilado
BSA - Albumina sérica bovina
DIC - Delineamento inteiramente casualizado
DNPH - 2,4 dinitrofenilhidrazina
DPPH - 2,2-difenil-1-picrilhidrazil
DPS - 4-4'-Dithiodipiridina
GuHCl - Hidrocloroeto de guanidina
MAP - Embalagem com atmosfera modificada
MDA - Malonaldeído
MRPs - Maillard Products
PROTOX - Proteína oxidada
SPME - Micro extração em fase sólida
TBARs - Substâncias reativas ao ácido tioarbitúrico
TCA - Ácido tricloroacético
TEP - Tetraetoxipropano
TFT - Teor de fenólicos totais
WOF - Warmed-over-flavour

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1 INTRODUÇÃO

As mudanças no estilo de vida das pessoas levaram a uma grande demanda por alimentos de conveniência, que apresentam baixo custo, alta disponibilidade no mercado e que podem ser consumidos sem preparação elaborada. A baixa disponibilidade de tempo para preparar a comida e a criação de equipamentos como o micro-ondas reforçam a crescente demanda por alimentos de conveniência. No entanto, as condições de processamento, aquecimento, armazenamento a frio e de reaquecimento destes tipos de alimentos são responsáveis pelo aumento da susceptibilidade dos componentes, principalmente de produtos cárneos, em sofrer degradação por oxidação, levando a uma deterioração notável da qualidade (BRUNNER, VAN DER HORST, SIEGRIST, 2010; BUCKLEY, COWAN, MCCARTHY, 2007; PEGG; SHAHIDI, 2012; SOLADOYE, et al., 2015).

As carnes e os produtos cárneos cozidos sofrem uma deterioração de sabor rápida e intensa durante o armazenamento a frio comumente descrito como warmed-over-flavour (WOF) (PEGG; SHAHIDI, 2012). Este sabor desagradável, indicado como responsável pela rejeição do consumidor e por perdas econômicas relevantes, está ligado à formação de compostos voláteis (como o pentanal, hexanal e alcadienos) derivados de lipídios específicos, os quais são facilmente percebidos pelo consumidor devido seus baixos limiares de detecção (JAYASENA et al., 2013). A oxidação destes lipídios é particularmente relevante em produtos de aves, devido ao alto grau de gordura insaturada e alta suscetibilidade às reações de oxidação (PEGG; SHAHIDI, 2012).

A oxidação de proteínas é uma reação em cadeia que leva à modificação química das proteínas alimentares com consequências negativas sobre o sistema alimentar. A formação de compostos carbonílicos, a degradação do triptofano, histidina, metionina e cisteína, perda de grupos sulfidrilas e formação de ligação cruzada são marcadores do dano químico de proteínas oxidadas (STADTMAN & LEVINE, 2003; ESTEVEZ; 2015). Em produtos cárneos, a oxidação de proteínas resulta em perda de qualidade nutricional, digestibilidade e funcionalidade proteica (CHEN; ZHAO; SUN, 2013; ESTEVEZ, 2015; ESTÉVEZ et al., 2011; LUND et al., 2011a; SOLADOYE et al., 2015). Além da perda do seu valor nutricional a oxidação de proteínas também afeta a textura dos produtos cárneos (LIU; XIONG; BUTTERFIELD, 2000). Durante o processo

oxidativo de proteínas, ligações cruzadas são formadas, o que aumenta a dureza de carnes e derivados (ESTÉVEZ; VENTANAS; CAVA, 2005; LUND et al., 2007). Além disso, o acúmulo de determinados produtos de oxidação de proteínas durante o processamento pode causar riscos à saúde dos consumidores devido ao potencial de toxicidade destas substâncias (SOLADOYE et al., 2015; ESTÉVEZ, 2015). A má absorção das proteínas oxidadas (PROTOX) podem gerar resíduos que após serem retidos no cólon levam a produção de metabólitos mutagênicos (fenóis e p-cresol), relatados no aumento do risco de câncer de cólon e colite ulcerativa (EVENEPOEL et al., 1998). Além deste efeito, Wang et al., (2013) relataram a evidência da absorção e efeito biológico negativo dos produtos da oxidação da lisina (AAS), reportado como um modelador potencial de homeostase da glicose em seres humanos levando a um aumento do risco de diabetes. Os produtos da oxidação da lisina (AAS e AAA) além de servirem como marcadores *in vivo* do estresse oxidativo, envelhecimento e doenças específicas, podem estar diretamente relacionados com a patogênese de desordens fisiológicas graves após a ingestão (ESTEVEZ et al., 2011; UTRERA et al., 2012, UTRERA & ESTEVEZ, 2013).

O uso de antioxidantes associado ao armazenamento em embalagens com atmosfera modificada (MAP) tem sido as principais estratégias da indústria para minimizar os efeitos negativos das oxidações lipídica e proteica na deterioração da qualidade dos produtos cárneos. Os antioxidantes sintéticos, tais como 2,3-terc-butil-4-hidroxianisol (BHA), di-terc-butil metil fenol (BHT) e terc-butil hidroquinona (TBHQ), são os mais utilizados em carnes com o objetivo de inibir as reações de oxidação (FASSEAS et al., 2008). Entretanto, devido ao potencial toxicológico dos antioxidantes, criou-se uma demanda por antioxidantes naturais na indústria da carne (KARRE; LOPEZ; GETTY, 2013). Os compostos provenientes de ervas aromáticas, especiarias, legumes, frutas e oleaginosas apresentam uma composição rica em flavonoides e compostos fenólicos (catequinas, carvacrol, procianidinas, ácidos benzoicos, entre outros), os quais contribuem para a capacidade antioxidante destes vegetais (FALOWO; FAYEMI; MUCHENJE, 2014).

No eixo das embalagens, o uso de MAP (normalmente composta por 70-80% de O₂ e 20-30% de CO₂) é um método amplamente utilizado para o armazenamento e conservação da carne, visando o aumento da vida de prateleira e a melhora na coloração dos cortes cárneos (ZAKRYS-WALIWANDER et al., 2012). No entanto, as

embalagens com altas concentrações de oxigênio podem ocasionar um aumento na oxidação de lipídios e proteínas (ZAKRYS et al., 2008). Diversos estudos abordam os efeitos de antioxidantes naturais no controle das oxidações lipídica e proteica (CICHOSKI et al., 2011; ESTÉVEZ; VENTANAS; CAVA, 2005, 2007; NIETO et al., 2013; UTRERA et al., 2015), entretanto poucos avaliam os efeitos combinados de antioxidantes naturais e MAP no controle destes processos oxidativos (JONGBERG et al., 2011; LARA et al., 2011; LUND et al., 2007). Assim, objetivou-se com este trabalho avaliar o impacto de três tipos de cozimento (imersão em água quente, forno e chapa), do tipo de embalagem (ar sintético e baixa concentração de oxigênio), do uso de antioxidante natural (extrato de *Quercus ilex* L.) e do reaquecimento em micro-ondas na deterioração físico-química, no valor nutritivo, na aceitação sensorial, na oxidação de lipídios e proteínas e na digestibilidade de proteínas em emulsionados tipo hambúrguer de frango prontos para consumo.

2 REVISÃO DE LITERATURA

2.1 ALIMENTOS PRONTOS PARA O CONSUMO

As mudanças no ritmo de vida da população dos centros urbanos e na estrutura tradicional das famílias associadas a evolução da tecnologia têm apresentado impacto direto na indústria mundial no que se refere a produção de bens de consumo. No setor alimentício, por exemplo, sabe-se que há uma tendência por produtos que apresentem conveniência e praticidade, em virtude, principalmente, da baixa disponibilidade de tempo para o preparo das refeições por parte dos consumidores que trabalham em tempo integral. Esta tendência tem estimulado uma demanda por alimentos prontos para o consumo, de fácil preparo, também chamados de produtos de conveniência. O consumo destes alimentos prontos ou semiprontos está associado à minimização de esforço físico e mental e a economia de tempo no preparo das refeições (MAN; FULLERTON, 1990).

Os produtos de conveniência são geralmente comercializados com maior frequência em redes de *fast food*. Nos Estados Unidos, por exemplo, cerca de 83% dos consumidores frequentam estabelecimentos de *fast food* pelo menos uma vez na semana (STATISTA, 2014). No Brasil, 31% da população consomem comida fora do domicílio semanalmente, devendo atingir 50% até 2020 (ABRASEL, 2012). De acordo com dados da última Pesquisa de Orçamentos Familiares (POF) realizada no período 2008-2009 pelo Instituto Brasileiro de Geografia e Estatística (IBGE, 2012), a média de gastos do brasileiro com a alimentação fora do domicílio para as famílias com renda *per capita* mensal de até mil reais correspondeu a 25% dos gastos totais. É provável que os gastos com este tipo de alimentação irão atingir 30% do salário do consumidor até 2020 (DONNA, 2010).

No Brasil, existe uma tendência para o consumo de produtos em pequenas porções (*snacking, finger food*), produtos embalados para consumo individual, produtos adequados para o consumo no trânsito ou em diferentes lugares e situações (BRASIL FOOD TRENDS 2020, 2010). Apesar desta demanda por alimentos prontos para o consumo, a população está cada vez mais preocupada com temas relacionados a saúde e bem-estar, o que tem desafiado a indústria de alimentos na

busca por produtos saudáveis, práticos, prontos para o consumo, que apresentem sabor e que a sua produção não tenha impacto negativo no meio ambiente.

É muito comum, na indústria de produtos cárneos o uso de emulsões. A estrutura básica de uma emulsão cárnea pode ser considerada uma mistura na qual os constituintes cárneos finamente divididos dispersam-se de modo análogo em uma dispersão de gordura em água onde, a fase descontínua é a gordura e a fase contínua é formada por uma solução aquosa de sais e proteínas. Nos produtos cárneos a emulsão é geralmente definida de acordo com sua aparência, já que parte da carne está finamente cominuída e não existe distinção entre as partes do produto (ORDÓÑEZ, 2005). A aplicação de tratamento térmico em produtos cárneos emulsionados tem o objetivo de estabilizar as múltiplas fases da massa cárnea e conferir ao produto final propriedades sensoriais aceitáveis. Normalmente os hambúrgueres não são definidos como produtos emulsionados, entretanto a Instrução Normativa nº 20, de 31 de julho de 2000 (BRASIL, 2000) não restringe sua classificação de acordo com o grau de cominuição da carne utilizada.

Dentre os produtos de conveniência mais consumidos, destaca-se o hambúrguer, o qual é definido pela Instrução Normativa nº 20, de 31 de julho de 2000 (BRASIL, 2000) como o produto cárneo industrializado obtido da carne moída dos animais de açougue, adicionado ou não de tecido adiposo e ingredientes, moldado e submetido a processo tecnológico adequado. O hambúrguer é um produto cárneo reestruturado que pode ser comercializado cru, semi-frito, cozido, frito, congelado ou resfriado, apresentando como ingrediente obrigatório a carne de animais de açougue e como ingredientes opcionais a gordura animal ou vegetal, água, sal, proteínas de origem animais ou vegetal, leite em pó, açúcares, maltodextrina, aditivos intencionais, condimentos, aromas e especiarias, vegetais, queijos e outros recheios. Com relação a sua composição físico-química, o hambúrguer deve apresentar até 23% de gordura e no mínimo 15% de proteína. O teor de carboidratos totais não deve ultrapassar 3%. Na Tabela 1 estão descritos os requisitos físico-químicos para o hambúrguer cru e cozido de acordo com a legislação brasileira (BRASIL, 2000). Nos Estados Unidos, o termo hambúrguer se refere ao bife de carne moída, fresco ou congelado, com ou sem adição de gordura e/ou condimentos, que não deve apresentar mais de 30% de gordura e não deve conter adição de água (ROMANS et al., 2000).

Tabela 1. Características físico-químicas do hambúrguer cru e cozido.

Componentes	Hambúrguer cru (%)	Hambúrguer cozido (%)
Gordura (máxima)	23,0	23,0
Proteína (mínima)	15,0	15,0
Carboidratos totais	3,0	3,0
Cálcio (máximo base seca)	0,10	0,45

Fonte: Brasil, 2000.

Segundo Alves (1999), o hambúrguer foi criado na cidade alemã de Hamburgo em meados do século XVII, onde o produto era tradicionalmente consumido cru. No entanto, de acordo com Smith (2006), a origem do hambúrguer é mais antiga, sendo introduzido na Alemanha pelos Tártaros Russos por volta do século XIV.

O hambúrguer foi introduzido nos Estados Unidos através de imigrantes que partiram de Hamburgo no século XIX. A partir disso, os americanos aperfeiçoaram a receita e tornaram o produto um dos símbolos da evolução e do atual estilo de vida norte-americano (ALVES, 1999).

2.2 REAÇÕES DE OXIDAÇÃO EM PRODUTOS CÁRNEOS

O processo de oxidação é definido como a transferência de elétrons de uma molécula que se oxida para outra que se reduz. Durante a oxidação, o agente oxidante pode abstrair um elétron na forma de átomos de hidrogênio de uma molécula susceptível através da formação de radicais livres pela ação das substâncias reativas de oxigênio (ROS) ou de nitrogênio (RNS) (BEKHIT et al., 2013).

Na célula viva, em condições fisiológicas normais, as reações de oxidação são fundamentais para obtenção eficiente da energia necessária para o funcionamento do organismo. Em contraste, a apoptose celular bem como o desenvolvimento de doenças crônicas e o envelhecimento também são acelerados por processos oxidativos que envolvem a formação de radicais livres (FIERS et al., 1999).

As transformações oxidativas têm sido associadas a efeitos deletérios na qualidade dos produtos alimentícios. Os tecnólogos de alimentos consideram a oxidação como um dos principais problemas relacionados a deterioração da qualidade da carne e produtos cárneos durante a estocagem (CONTINI et al., 2014). Após o

abate dos animais e durante os eventos *post mortem*, ocorre um desequilíbrio entre os componentes pro-oxidantes e antioxidantes da célula muscular devido as alterações bioquímicas decorrentes da conversão do músculo em carne, promovendo a degradação oxidativa de lipídeos e proteínas (MORRISSEY et al., 1998).

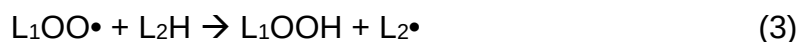
2.2.1 Oxidação lipídica em carnes e derivados

A oxidação de lipídeos consiste na reação em cadeia via radicais livres entre o oxigênio e lipídeos insaturados. Os mecanismos químicos envolvidos nesta reação já são consolidados na literatura e podem ser divididos em três etapas: iniciação, propagação e terminação.

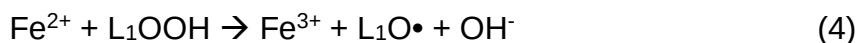
Apesar da oxidação lipídica ser considerada como um processo auto catalítico, ou seja, aquele que uma vez iniciado se propaga de modo independente, é necessário um iniciador ou catalisador que remova um elétron do lipídeo ou do oxigênio, dando origem a radicais livres (ESKIN; SHAHIDI, 2015). Durante a fase de iniciação, na presença de catalisadores ou ROS, ocorre a formação de radicais livres pela abstração de um átomo de hidrogênio de lipídeos insaturados (reação 1). De acordo com Choe; Min (2005), o radical hidroxil (OH•) apresenta potencial de redução elevado (2300 mV), sendo o principal responsável pela iniciação da oxidação lipídica.



Em contato com o oxigênio, o radical lipídico (alquila) formado se converte em outros radicais (reação 2) com maior poder oxidante (peroxil, hidroxil, etc.), dando início a fase de propagação. Nesta etapa, o radical formado abstrai outro hidrogênio de um lipídeo insaturado distinto (reação 3), iniciando a reação em cadeia com formação de mais radicais livres e hidroperóxidos (LOOH) como produto primário da oxidação dos lipídeos.



Os hidroperóxidos formados na fase de propagação também podem reagir com metais de transição (Fe^{2+} e Cu^+), formando radicais peroxil e alcóxil, os quais apresentam reatividade intermediária (reação 4). A degradação de hidroperóxidos acelerada por íons metálicos é denominada de reação de Fenton (FENTON, 1984)



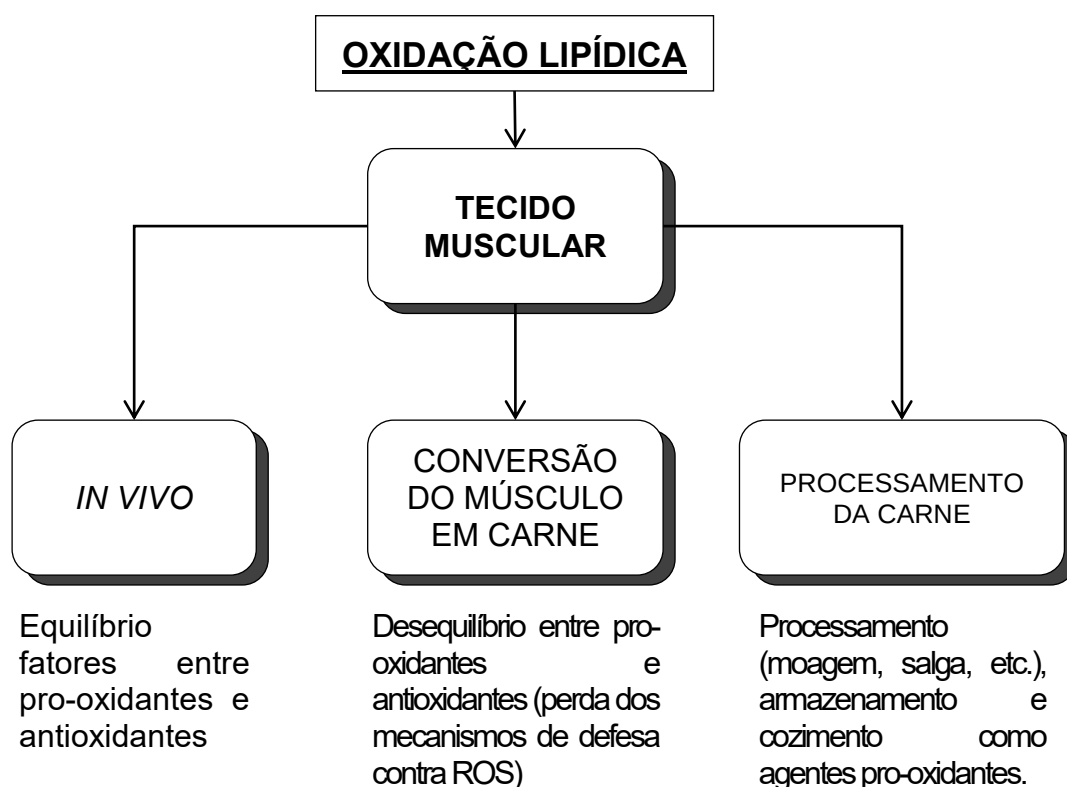
No estágio final da oxidação (terminação), os radicais livres formados se rearranjam e reagem entre si para formar produtos estáveis por mecanismos distintos. Nesta fase, pode haver a recombinação entre dois radicais alquila com a formação de polímeros de alcanos (reação 5). Os radicais peroxil ainda podem reagir com outros compostos para formar peróxidos de alquila (reação 6).



Por fim, radicais alcóxil também podem reagir com lipídeos insaturados, formando álcoois, cetonas, éteres e aldeídos insaturados como o malonaldeído (MDA), os quais são considerados como produtos secundários da oxidação lipídica. Apesar da terceira fase da oxidação lipídica ser denominada de terminação, o processo oxidativo não é totalmente paralisado, pois há a formação, ainda que lenta e em baixa concentração, de radicais reativos que podem reagir com lipídeos insaturados (ESKIN; SHAHIDI, 2015).

A oxidação de lipídeos pode se manifestar em três estágios distintos no tecido muscular. Segundo Morrissey et al. (1998), a degradação oxidativa de lipídeos pode ocorrer no animal vivo, durante a conversão do músculo em carne e durante o processamento após a transformação do músculo em carne (FIGURA 1).

Figura 1. Oxidação lipídica no tecido muscular.



O processo de oxidação lipídica no músculo vivo é importante principalmente para obtenção de energia para o funcionamento celular em condições fisiológicas normais. Nesta fase, existem vários mecanismos limitantes da exposição lipídica aos agentes pro-oxidantes. No entanto, quaisquer alterações no equilíbrio entre os fatores pro-oxidantes e antioxidantes podem acelerar as reações de oxidação lipídica e promover o surgimento de doenças crônicas e envelhecimento (FIERS et al., 1999).

As alterações bioquímicas que ocorrem durante a conversão do músculo em carne proporcionam um desequilíbrio entre os componentes pro-oxidantes e antioxidantes do tecido muscular, causando oxidação descontrolada da fração fosfolipídica insaturada das membranas celulares. Segundo Estévez; Morcuende; Ventanas (2008), o metabolismo anaeróbico, a parada do sistema de circulação sanguínea e do aporte de nutrientes, e a perda dos mecanismos de proteção enzimática associados aos eventos pós abate tais como a queda brusca do pH, temperatura da carcaça e o uso de técnicas de amaciamento (i.e., estimulação elétrica) favorecem a descompartmentalização celular, liberando íons metálicos (principalmente ferro) responsáveis pela catálise das reações de oxidação lipídica.

A liberação do ferro proveniente da mioglobina, hemoglobina e ferritina também ocorre após as etapas de processamento (salga, moagem, etc.), estocagem e cozimento da carne e dos produtos cárneos, o que promove alterações intensas de cor, aroma e sabor que são bastante perceptíveis pelo consumidor. Durante o armazenamento, fatores como calor, umidade, oxigênio, presença de luz, metais, enzimas e pigmentos podem promover a oxidação de lipídeos, gerando compostos prejudiciais à saúde humana, tais como malonaldeídos e óxidos de colesterol, os quais possuem atividade carcinogênica comprovada (SOUZA; ARTHUR; CANNIATTI-BRAZACA, 2007; BAGGIO; BRAGAGNOLO, 2006).

A influência do processo de cocção na velocidade da oxidação lipídica depende da intensidade do tratamento térmico. Segundo Ladikos; Lougovois (1990), a promoção da oxidação lipídica ocorre apenas em temperaturas de cozimento abaixo de 80 °C. Em temperaturas de cocção elevadas (> 80 °C) e/ou longos períodos de cozimento, ocorre diminuição nos marcadores da oxidação lipídica. Os referidos autores explicam que este fenômeno pode ser atribuído ao desenvolvimento de substâncias com potencial antioxidante a partir da formação de produtos da reação de Maillard em altas temperaturas e/ou longos períodos de cozimento. De acordo com

Enser (1987), a oxidação lipídica que ocorre durante o cozimento pode dar origem a compostos responsáveis pela formação do aroma desejável na carne cozida.

O aroma reaquecido (*warmed-over flavour*) é definido como aquele formado durante o reaquecimento de carnes cozidas estocadas em condições de refrigeração (TIMS; WATTS, 1958). Com a evolução da tecnologia e a mudança na estrutura familiar, este tipo de problema tornou-se frequente em virtude do crescimento da produção e da demanda por produtos prontos para o consumo ou de conveniência, os quais são geralmente reaquecidos antes do consumo.

As consequências da oxidação lipídica para a qualidade da carne dependem basicamente da composição lipídica da matéria prima e das condições de processamento e estocagem do produto final, sendo relacionadas com a perda da qualidade de seus produtos no que se refere principalmente às alterações de aroma e sabor com desenvolvimento do ranço. Além disso, pode haver a degradação da cor da carne em detrimento da oxidação do pigmento oximioglobina (vermelho) com formação de metamioglobina (marrom) (JOHNS; BIRKINSHAW; LEDWARD, 1989). Embora a oxidação lipídica promova a produção de aromas e sabores indesejáveis na maioria dos produtos cárneos, os compostos voláteis oriundos desta reação são essenciais para o desenvolvimento do aroma e sabor característicos de produtos dessecados e curados, tais como presuntos e embutidos curados, e alguns embutidos fermentados (RUIZ. MURIEL; VENTANAS, 2002; PEARSON; LOVE; SHORLAND, 1977). Os produtos formados após a oxidação de lipídeos têm sido caracterizados como tóxicos e associados com o desenvolvimento de processos deteriorantes da saúde humana. Peróxidos e óxidos de colesterol podem estar envolvidos no desenvolvimento de tumores e arteriosclerose, enquanto que o malonaldeído tem sido caracterizado como agente mutagênico e relacionado com a formação de nitrosaminas (BAGGIO; BRAGAGNOLO, 2006; PERSON et al., 1983).

2.2.2 Oxidação proteica em carnes e derivados

A oxidação de lipídeos e dos pigmentos são reconhecidas como as principais causas da deterioração da qualidade da carne fresca e processada durante a estocagem. Sabe-se que a oxidação das proteínas nos tecidos vivos tem papel fundamental no desenvolvimento de doenças degenerativas ligadas ao

envelhecimento tais como Alzheimer (DAVIES, 2003; STADTMAN; BERLETT, 1997). No entanto, o fato das proteínas serem alvos das substâncias reativas de oxigênio (ROS) foi ignorado por várias décadas, enquanto que o efeito da degradação oxidativa de lipídeos na qualidade da carne foi extensivamente estudado (CONTINI et al., 2014; BEKHIT et al., 2013; CHAIJAN, 2008; JOHNS; BIRKINSHAW; LEDWARD, 1989).

Os estudos envolvendo a oxidação de proteínas tiveram início com pesquisas relacionadas às ciências médicas e à biologia humana (LUND; HVIID; SKIBSTED, 2007). A descoberta de que as proteínas cárneas eram susceptíveis às reações oxidativas promoveu o aumento do interesse por estudos relacionados aos efeitos da oxidação de proteínas na qualidade dos alimentos no início dos anos 90. No entanto, o significado científico e tecnológico da degradação oxidativa de proteínas ainda não foi totalmente elucidado. Apesar do avanço nos estudos relacionados aos impactos da oxidação de proteínas no envelhecimento e no desenvolvimento de doenças degenerativas, existe uma lacuna no conhecimento sobre bioquímica de alimentos devido aos poucos estudos envolvendo os efeitos da oxidação proteica em produtos alimentícios. Segundo Lund et al. (2011), o atraso na evolução das pesquisas relacionadas a oxidação proteica de alimentos deve-se a ausência de metodologias específicas para identificar e quantificar a oxidação das proteínas associada ao fato da degradação oxidativa de lipídeos ser considerada, juntamente com a deterioração microbiológica, como a principal causa da perda de qualidade dos alimentos.

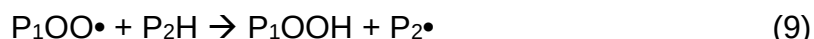
Por definição, a oxidação proteica é descrita como um processo de reações em cadeia mediadas por radicais livres (LUND et al., 2011). No entanto, os mecanismos desta reação são bastante complexos e existe uma quantidade considerável de compostos que podem ser formados (DAVIES; DEAN, 2003). Assim como ocorre com os lipídeos, os mecanismos químicos envolvidos nas reações de oxidação das proteínas podem ser divididos em três etapas: iniciação, propagação e terminação.

Na fase inicial, ocorre a abstração de um átomo de hidrogênio da molécula proteica vulnerável por um radical livre, levando a formação de um radical proteico (reação 7). Na presença de oxigênio molecular, o radical proteico é convertido em radical peroxila (reação 8). Os tipos de compostos formados ao final da oxidação proteica dependem de como a reação se inicia (LUND et al., 2011). Várias ROS têm sido reconhecidas como agentes pro-oxidantes iniciadores da oxidação em proteínas, tais como radicais superóxido ($O_2^{\bullet-}$) e hidroperoxila ($HO_2^{\bullet}$) e espécies não radicais

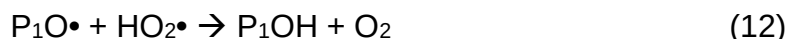
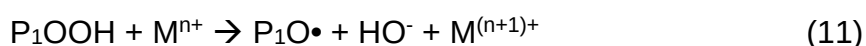
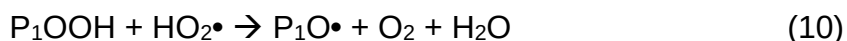
como o peróxido de hidrogênio (H_2O_2) e os hidroperóxidos (ROOH). A cadeia polipeptídica e os grupos funcionais localizados na cadeia lateral de aminoácidos são os principais alvos dos radicais com potencial oxidativo.



Na etapa de propagação, o radical peroxila formado pode abstrair outro átomo de hidrogênio de uma molécula proteica adjacente, promovendo a formação de peróxido de alquila e de mais um radical proteico (reação 9).



O peróxido de alquila pode reagir com outras ROS e metais de transição formando radicais alcóxila (reações 10 e 11). Este radical pode atuar abstraindo um átomo de hidrogênio de outra molécula proteica vulnerável ou reagir com outros radicais livres dando origem a derivados hidroxilados (reação 12).

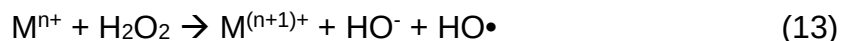


Na fase de terminação, o radical proteico pode reagir com outro radical proteico formando um agregado proteico (ligação cruzada), ou com produtos secundários da oxidação lipídica (malonaldeídos), dando origem aos compostos carbonílicos. Também pode haver a formação de fragmentos peptídicos quando os compostos pro-oxidantes atacam diretamente a cadeia polipeptídica (ESTÉVEZ, 2011).

A carbonilação proteica é uma reação não enzimática irreversível que requer a presença de radicais livres e metais de transição, envolvendo a formação de compostos carbonílicos induzidos principalmente pelo estresse oxidativo de proteínas (BERLETT; STADTMAN, 1997). De acordo com Estévez (2011), os compostos carbonílicos podem ser formados por quatro vias distintas: (i) oxidação direta da cadeia lateral da lisina, prolina, treonina e arginina, (ii) glicação não enzimática na presença de açúcares redutores, (iii) clivagem oxidativa da cadeia polipeptídica e (iv) ligação covalente entre o radical proteico e produtos secundários da oxidação lipídica tais como malonaldeído (MDA) e 4-hidroxi-2-nonenal (HNE).

A oxidação direta da cadeia lateral de aminoácidos tem sido reconhecida como a principal via para a carbonilação proteica (ESTÉVEZ, 2011; SHACTER, 2000). A formação de compostos carbonílicos derivados das cadeias laterais de lisina, treonina,

arginina e prolina tem sido atribuída ao desenvolvimento de compostos reativos intermediários tais como o radical hidroxil ($\text{HO}\bullet$) através da degradação do peróxido de hidrogênio (H_2O_2) pela ação de metais de transição (ferro, cobre e manganês) via reação de Fenton (reação 13).



Os aminoácidos sulfurados são os mais susceptíveis às reações de oxidação porque os átomos de enxofre são bastante reativos devido a elevada quantidade de elétrons presentes no ânion de enxofre (SHACTER, 2000). Resíduos de triptofano também são facilmente oxidados na presença de metais de transição devido ao conjunto de anéis aromáticos. Além disso, certos aminoácidos tais como metionina e triptofano possuem atividade antioxidante, podendo atuar como protetores dos outros aminoácidos se ligando a substância pro-oxidante e sendo, muitas vezes, os primeiros a serem oxidados (LEVINE et al., 1999).

Dentre os fatores que influenciam a velocidade da oxidação proteica na carne, pode-se destacar a presença de metais de transição, lipídeos oxidados e inibidores bem como o nível de mioglobina, pH, temperatura, atividade de água, luz e radiação, além da composição aminoacídica da proteína (ESTÉVEZ; MORCUENDE; VENTANAS, 2008). Metais de transição como íons de ferro e cobre podem favorecer a formação de radicais hidroxilados via reação de Fenton e a quantidade de mioglobina tem relação positiva com os níveis de ferro heme presentes no sistema. Como já foi explicitado anteriormente, lipídeos oxidados podem interagir com os radicais proteicos dando origem a compostos carbonílicos. O pH está associado com a funcionalidade da proteína, logo, valores extremos de pH tendem a favorecer as reações de oxidação proteica. A presença de luz, radiação e temperaturas elevadas são fatores pro-oxidantes que promovem a energia necessária para a fase de iniciação das reações de oxidação proteica.

Na sociedade moderna, a carne é quase sempre cozida antes do consumo e o tratamento térmico empregado pode acelerar as reações de oxidação pelo aumento da produção de radicais livres e diminuição dos mecanismos antioxidantes de proteção da carne (GATELLIER et al., 2010). O impacto do cozimento/tratamento térmico nos parâmetros de oxidação dos lipídeos já está consolidado (LADIKOS; LOUGOVOIS, 1990; ENSER, 1987). No entanto, pouco se sabe a respeito do efeito dos métodos de cocção na oxidação proteica. Segundo Traore et al. (2012), o

processo de cozimento atua como promotor da geração de substâncias pro-oxidantes (ROS), aumentando a chance da oxidação de proteínas. Além disso, a atividade dos sistemas antioxidantes da carne (i.e., glutathione peroxidase e catalase) é reduzida pela aplicação de calor. Após o cozimento, a concentração de ferro heme da carne diminui e o teor de ferro não heme aumenta, o que favorece a iniciação da oxidação proteica via reação de Fenton (ESTÉVEZ, 2011). Estudos têm demonstrado que o processo de cozimento promove a desnaturação térmica da proteína através de mudanças estruturais que aumentam a hidrofobicidade superficial da proteína e induzem a formação de agregados proteicos, levando a uma diminuição da digestibilidade da proteína (PROMÉYRAT et al., 2010; SANTÉ-LHOUTELLIER et al., 2008). Segundo Evenepoel et al. (1998); Geypens et al. (1997), diminuições na taxa de digestão proteica podem ter impacto negativo na saúde humana devido ao fato de que proteínas não hidrolisadas podem ser fermentadas na flora intestinal com a formação de compostos mutagênicos (fenol e *p*-cresol), aumentando o risco de câncer de cólon.

De acordo com Lund et al. (2011), as consequências da oxidação proteica na qualidade da carne e de seus derivados têm sido associadas com alterações na solubilidade e funcionalidade das proteínas em termos de poder de geleificação e emulsificação e/ou capacidade de retenção de água (CRA), o que pode ter efeito direto no rendimento após a cocção e na suculência da carne. Além disso, as modificações oxidativas das proteínas também podem promover a perda de aminoácidos essenciais e diminuir a digestibilidade proteica, afetando a qualidade nutricional da carne. No que se refere aos aspectos sensoriais, estudos recentes têm reportado que a oxidação proteica pode afetar as características de textura e sabor da carne (ESTÉVEZ, 2011). Em termos de maciez, a oxidação proteica pode promover a inativação de enzimas proteolíticas responsáveis pelo amaciamento *post mortem* e induzir a formação de ligações cruzadas entre proteínas miofibrilares via pontes dissulfeto, causando enrijecimento do tecido muscular (KIM et al., 2010; LUND et al., 2007). Fuentes et al. (2010); Ventanas et al. (2007) sugeriram que a formação de compostos carbonílicos de origem proteica podem ter efeito significativo no desenvolvimento do aroma e sabor característicos de produtos dessecados e curados. No entanto, os mecanismos deste fenômeno ainda não foram totalmente elucidados.

2.3 CONTROLE DA OXIDAÇÃO LIPÍDICA E PROTEICA EM PRODUTOS CÁRNEOS

Conforme explicitado anteriormente, as reações de oxidação podem acometer tanto os lipídeos quanto as proteínas da carne, promovendo, em sua maioria, alterações indesejáveis sob o ponto de vista tecnológico, nutricional e sensorial do alimento. Contudo, existem diferenças quanto aos mecanismos químicos envolvidos entre a oxidação lipídica e proteica. A fase de iniciação da oxidação lipídica ocorre predominantemente pelo ataque às duplas ligações de lipídeos insaturados enquanto que a molécula proteica contém grande quantidade de diferentes grupos funcionais que podem ser afetados pela oxidação a depender da composição aminoacídica e da conformação da proteína (LUND, 2007). Por isso, o controle simultâneo da oxidação de lipídeos e proteínas tem se tornado um desafio para a indústria de alimentos.

Com a evolução da sociedade, o aumento do consumo de alimentos pré-prontos e de conveniência fez com que houvesse o reconhecimento da importância do controle da oxidação nestes produtos. Em virtude do fato de que inicialmente acreditava-se que os mecanismos químicos envolvidos nas reações de oxidação lipídica e proteica eram impactados pelos mesmos fatores, a maioria das estratégias desenvolvidas para o controle da oxidação de lipídeos foram testadas contra oxidação proteica. No entanto, estudos posteriores demonstraram que as estratégias antioxidantes comprovadamente eficazes contra a oxidação lipídica não tiveram efeito nas proteínas cárneas ou até mesmo atuaram como agentes pró-oxidantes nas mesmas (ESTÉVEZ, 2011).

De acordo com a literatura, lipídeos e proteínas podem ser protegidos contra as reações de oxidação através de estratégias nutricionais e tecnológicas. As estratégias nutricionais estão relacionadas com a suplementação da ração animal com compostos que possuam atividade antioxidante (vitamina E, vitamina C, tocoferol e carotenoides por exemplo) e/ou modificação da alimentação dos animais visando alterar a composição lipídica da carne em termos de ácidos graxos. A estratégia tecnológica se refere basicamente ao controle dos fatores ambientais que limitam a oxidação da carne.

Sabe-se que a alimentação animal tem influência marcante na estabilidade oxidativa da carne. Por isso, o enriquecimento da carne com substâncias naturais que possuam atividade antioxidante tem sido o foco dos pesquisadores na área de ciência

e tecnologia de alimentos (SMET et al., 2008; VENTANAS et al., 2006; BATIFOULIER et al., 2002; MERCIER et al., 1998; LOPEZ-BOTE et al., 1997). O uso de suplementação na dieta de animais de corte visando o controle da oxidação lipídica e proteica tem sido uma estratégia bastante utilizada na avicultura. Segundo Batifoulier et al. (2002), o uso de vitamina E na suplementação da alimentação de perus teve efeito protetor contra a oxidação lipídica da carne enquanto que não foi observado efeito significativo na formação de compostos carbonílicos oriundos de proteínas. A carne pode ser enriquecida com antioxidantes naturais, tais como o alfa-tocoferol (200 mg/kg ração), inseridos na alimentação do animal através da ração (LOPEZ-BOTE et al., 1997). A suplementação da dieta animal com alfa-tocoferol promoveu a diminuição da formação de compostos carbonílicos na carne bovina (ROWE et al., 2004), suína (VENTANAS et al., 2006) e de peru (DEAN et al., 1991) durante o armazenamento. A composição de ácidos graxos da carne de animais monogástricos também pode ser modificada através da dieta visando minimizar a oxidação lipídica (VENTANAS et al., 2006). Nestes casos, objetiva-se a redução da quantidade de ácidos graxos poli-insaturados (PUFA) da carne. Segundo Lund et al. (2011), a suplementação da dieta do animal com tocoferol e carotenoides parece ser efetiva na inibição da oxidação proteica enquanto a modificação da composição de ácidos graxos apresenta efeitos negligenciáveis na degradação oxidativa de proteínas. Por serem ácidos graxos essenciais não produzidos pelo organismo, a diminuição dos valores de PUFA nos produtos cárneos durante a evolução do processo oxidativo lipídico o valor nutricional destes produtos pode ser diminuído. Uma baixa ingestão destes ácidos graxos poli-insaturados pode ter importantes consequências sobre a saúde humana.

As estratégias tecnológicas para o controle da oxidação lipídica e proteica em carnes e derivados baseiam-se na inserção de substâncias com atividade antioxidante na formulação dos produtos e/ou redução da exposição da carne ao oxigênio molecular através do uso de embalagens com atmosfera modificada (ESTÉVEZ, 2011; LUND, 2011). No entanto, as estratégias antioxidantes para proteção dos lipídeos nem sempre se aplicam as proteínas musculares.

A adição de compostos fenólicos provenientes de extratos vegetais tem sido extensivamente utilizada no controle da oxidação lipídica em produtos cárneos (GANHÃO; MORCUENDE; ESTÉVEZ, 2010; VILJANEN; KIVIKARI; MERCIER et al., 1998). A inibição da oxidação lipídica previne a degradação oxidativa das proteínas

por minimizar a formação de compostos secundários da oxidação lipídica e, consequentemente, a sua interação com proteínas para formação de compostos carbonílicos.

Uma grande variedade de compostos fenólicos oriundos de plantas tem apresentado atividade antioxidante em produtos cárneos. Estes compostos podem ser classificados em substâncias hidrofóbicas e hidrofílicas (SHAHIDI; WANASUNDARA, 1992). Em sistemas modelo, os fenólicos hidrofílicos têm sido reportados como inibidores da oxidação lipídica e proteica (BARON et al., 2005). De acordo com Estévez; Heinonen (2010), os compostos fenólicos podem proteger as proteínas miofibrilares contra as reações oxidativas por dois mecanismos distintos: (i) atuando como agentes quelantes de íons metálicos, inativando o efeito pro-oxidante do ferro não-heme e (ii) se ligando aos radicais hidroxila formados pela degradação de hidroperóxidos via reação de Fenton. No entanto, na presença de metais de transição tais como ferro e cobre, determinados compostos fenólicos podem sofrer o processo de auto-oxidação, levando a formação de quinonas, as quais apresentam atividade de amino-oxidase. De acordo com este mecanismo, as quinonas de compostos fenólicos podem catalisar a desaminação oxidativa de aminoácidos susceptíveis, ocasionando a formação de semialdeídos (ESTÉVEZ; HEINONEN, 2010).

A interação entre os polifenóis e grupos sulfidril (SH) da carne não foi completamente elucidada. Em carne de pescado, por exemplo, o ácido cafeico demonstrou efeito protetor na oxidação de grupos sulfidril, mas aumentou a formação de compostos carbonílicos (EYMARD; BARON; JACOBSEN, 2009).

O efeito dos compostos fenólicos de plantas contra a oxidação proteica baseia-se em interações específicas estabelecidas através de ligações covalentes e não covalentes entre os compostos fenólicos e as proteínas (ESTÉVEZ, 2011). Segundo Kroll; Rawell (2001), estas interações dependem da quantidade e do estado químico do composto fenólico e do tamanho, conformidade e da carga global da proteína.

A modificação da atmosfera das embalagens visando a proteção contra as reações oxidativas em produtos cárneos também tem se apresentado eficiente (CONTINI et al., 2014; KIM et al., 2010; LUND et al., 2007; LUND; HVIID; SKIBSTED, 2007). O sistema de embalagem tem as funções básicas de conter, proteger e viabilizar o transporte dos produtos. Com a evolução da sociedade e da tecnologia, tornou-se cada vez mais frequente a busca por embalagens ativas e biodegradáveis.

No âmbito da indústria da carne, o desenvolvimento de novos tipos de embalagens para a manutenção da qualidade dos produtos ao longo do armazenamento em termos de controle da oxidação lipídica e deterioração microbiológica tem sido o foco dos produtores. Neste segmento, destaca-se o uso de embalagem com atmosfera modificada (EAM), que consiste na remoção (vácuo) e/ou substituição da atmosfera em torno do produto com posterior selagem com uso de materiais que possuam barreiras contra a entrada e saída de vapor de água (MCMILLIN et al., 1999).

O uso de embalagens a vácuo pode ser considerado como atmosfera modificada. Esta técnica baseia-se na remoção do oxigênio da embalagem antes da selagem. Dentre os principais benefícios do uso de embalagem a vácuo, estão o controle da oxidação lipídica e do desenvolvimento de microrganismos deteriorantes aeróbios. No entanto, o uso de embalagem a vácuo apresenta impacto negativo em determinadas características da carne fresca, sobretudo no que se refere aos atributos sensoriais, tendo em vista que atmosferas com elevada concentração de oxigênio preserva a cor vermelha e brilhante da carne. Por isso, pesquisadores têm evidenciado a substituição do oxigênio por mistura de gases inertes tais como nitrogênio (N_2) ou gás carbônico (CO_2) para a conservação da carne e de seus derivados (LUND; HVIID; SKIBSTED, 2007). Contudo, os efeitos deste tipo de embalagem na carbonilação proteína são inconclusivos. Lund et al. (2007) observaram um aumento da carbonilação proteica em produtos de carne bovina quando embalada em atmosfera com elevada concentração de oxigênio (80% O_2 /20% N_2 vs 100% N_2) enquanto Lund; Hviid; Skibsted (2007) reportaram que a carne suína parece não ser afetada pelas condições de embalagem, sugerindo que o efeito da embalagem na carbonilação proteica é variável e depende da espécie animal.

De acordo com Estévez (2011), a carne embalada em atmosfera com elevada concentração de oxigênio apresenta risco elevado para as reações de oxidação. No entanto, a fase de iniciação para formação de compostos carbonílicos de proteínas necessita da presença de agentes pro-oxidantes (metais de transição, lipídeos oxidados, etc.) ou processamentos (irradiação, cozimento, etc.) capazes de ativar o oxigênio molecular e gerar ROS.

2.3.1 Fruto do gênero *Quercus*

Na Península Ibérica a paisagem é caracterizada pela proliferação de madeira da floresta pelo gênero *Quercus*, onde destacam-se a azinheira (*Quercus ilex* L.), o sobreiro (*Quercus suber* L.) e o carvalho Português (*Quercus faginea* L.). Estas espécies são caracterizadas por um crescimento lento, de modo que um carvalho começa a produzir uma quantidade apreciável de bolotas de 20-25 anos e atinge a produção máxima a cerca de 100 anos (DAZA, 1996).

A azinheira é uma das árvores mais características da floresta mediterrânea peninsular. É uma árvore grande com uma copa densa, podendo chegar a 27 metros de altura, embora geralmente não supere os 15 metros nos sistemas adensados devido à poda. Dotado de grande robustez, resistência e longevidade, adapta-se a condições climáticas adversas e solos pobres, rejeitando umidade excessiva. Seu fruto é a bolota, oblongo ou ovóide que está inserido em uma cúpula hemisférica com casca rígida e lisa. No início da frutificação apresenta cor verde e ao final do processo de maturação apresenta cor castanho escuro (PULIDO et al., 2004). A fruta tem um sabor doce que é muito apreciado por animais, como os porcos ibéricos, especialmente durante o período de pasto nas montanhas. De acordo com Saenz (1967), o sabor doce da bolota era o ponto-chave na aceitação para consumo humano no passado.

Quanto à composição nutricional das bolotas, as diferentes espécies de *Quercus* apresentam muita variação nos teores de proteínas, carboidratos, gorduras, vitaminas e minerais. Em vários estudos são relatadas diferenças na composição da bolota, dependentes do ano e das áreas geográficas em que os frutos foram colhidos. De modo geral, a composição físico-química das bolotas do gênero *Quercus ilex* apresentam 53,9-68,7% de matéria seca, 4,3-6% de proteína bruta, 5-9% de gordura, 2,5-6% de fibra bruta e 1,2-2,5% de minerais. Entre os ácidos graxos encontrados o ácido oleico monoinsaturado é o mais abundante (52,2-66,1%). Do teor total de lipídeos destaca-se também o conteúdo de vitamina E, que possui atividade antioxidante, principalmente o γ -tocoferol e α -tocoferol (CAVA et al., 1997; LÓPEZ-BOTE, 1998; CAVA et al., 1999; TEJEDA et al., 2002; FERNANDEZ et al., 2005; DAZA et al., 2005; REY et al., 2006; DAZA et al., 2007; TEJERINA et al., 2012).

As bolotas do gênero *Quercus* são um dos principais recursos alimentares da pastagem e representam o elemento-chave na alimentação de animais selvagens e domésticos. Vazquez (1998) enumera 9 espécies de grandes mamíferos, 11 espécies de pequenos mamíferos e 6 espécies de aves entre os principais consumidores dessas bolotas. Entre os consumidores domésticos, seu consumo é citado na criação de suínos ibéricos e na fase de engorda (PULIDO et al., 2004; RODRIGUEZ-ESTEVEZ et al., 2008). De acordo com estimativas de Dobao et al., (1988), durante a fase de engorda, o suíno consome de 6-10 kg de bolotas e 1-1,5 kg de grama por dia. Esta dieta tem um efeito positivo na qualidade da carne e no seu perfil de ácidos graxos (TEJEDA et al, 2002, DAZA et al, 2005; REY et al, 2006. VENTANAS et al, 2007, TEJERINA et al, 2012).

Vários pesquisadores enfatizam que a importância das diferentes espécies de bolotas não está apenas no seu papel como uma fonte importante de nutrientes, mas também de compostos antioxidantes (LEE et al, 1992; MAMEDOVA et al, 1993; CANTOS et al, 2003; RAKIE et al, 2007; TEJERINA et al, 2012).

3 MATERIAL E MÉTODOS

3.1 DELINEAMENTO EXPERIMENTAL

O trabalho experimental foi realizado no Laboratório de Tecnologia de Carnes do Departamento de Tecnologia de Alimentos, Faculdade de Veterinária da Universidade de Extremadura, Cáceres, Espanha, por meio do Programa de Doutorado Sanduíche no Exterior (PDSE/CAPES - processo número 8853/2014-02); durante o período de novembro de 2014 a maio de 2015 (6 meses).

Os estudos de oxidação lipídica e proteica dos emulsionados de frango envolveram dois experimentos: i) efeito de diferentes métodos de cozimento, armazenamento refrigerado e reaquecimento em micro-ondas na evolução do processo oxidativo de emulsionados de frango (Figura 2); ii) influência do uso de antioxidante natural, embalagem com atmosfera modificada, armazenamento refrigerado e reaquecimento em micro-ondas na qualidade de emulsionados de frango (Figura 3).

Figura 2: Delineamento experimental para avaliação do processo oxidativo de emulsionado tipo hambúrguer de frango

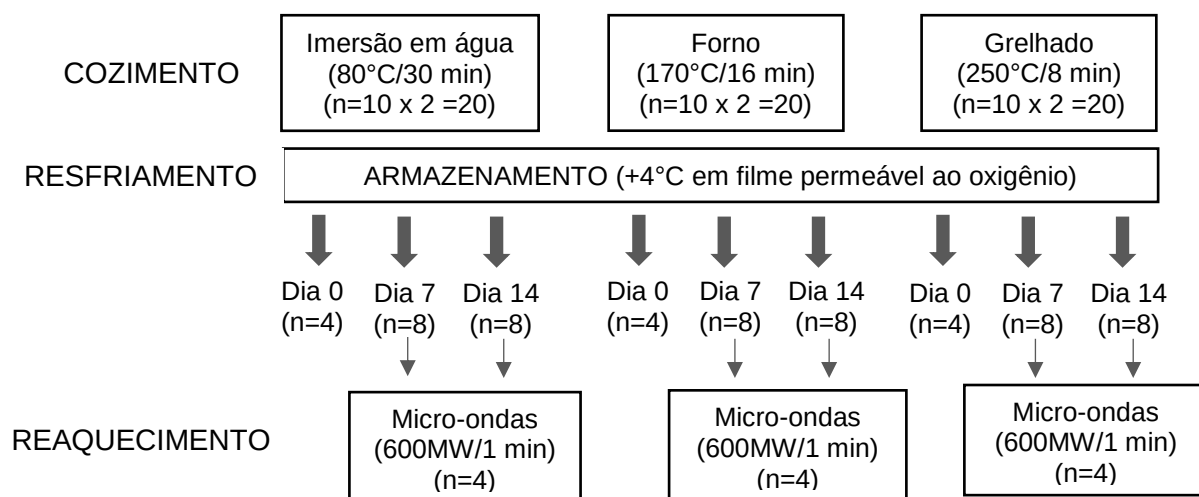
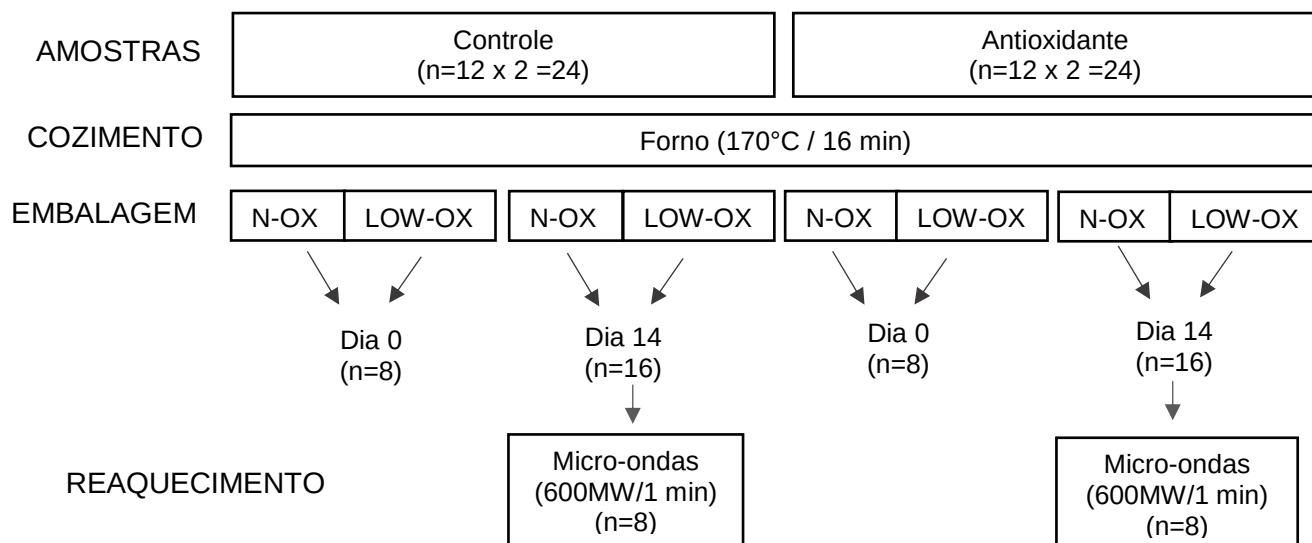


Figura 3: Delineamento experimental para avaliação do uso de antioxidante natural em emulsionado tipo hambúrguer de frango



N-OX: Embalagem com atmosfera normal ($21\% \text{O}_2$, $0,5\% \text{CO}_2$, $78,5\% \text{N}_2$)

LOW-OX: Embalagem com atmosfera modificada (impermeável ao O_2 - $8\% \text{O}_2$, $27\% \text{CO}_2$, $65\% \text{N}_2$)

No experimento 1 utilizou-se um delineamento inteiramente casualizado (DIC) sob um esquema fatorial 3×3 , analisando-se três métodos de cozimento e três tempos de armazenamento. O efeito do reaquecimento em micro-ondas em dois tempos de armazenamento também foi avaliado, totalizando 15 tratamentos (com 4 unidades experimentais cada), mais um lote de amostras cruas (com 4 unidades experimentais) para caracterização do produto.

No experimento 2 também foi utilizado um DIC sob um esquema fatorial $2 \times 2 \times 2$, analisando-se o uso de antioxidante natural, armazenamento e tipo de embalagem totalizando 8 tratamentos (com 4 unidades experimentais cada), mais um lote de amostras cruas (com 8 unidades experimentais) para caracterização dos produtos. O efeito do reaquecimento em micro-ondas também foi avaliado adicionando dois tratamentos (com 4 unidades experimentais cada) para serem avaliados.

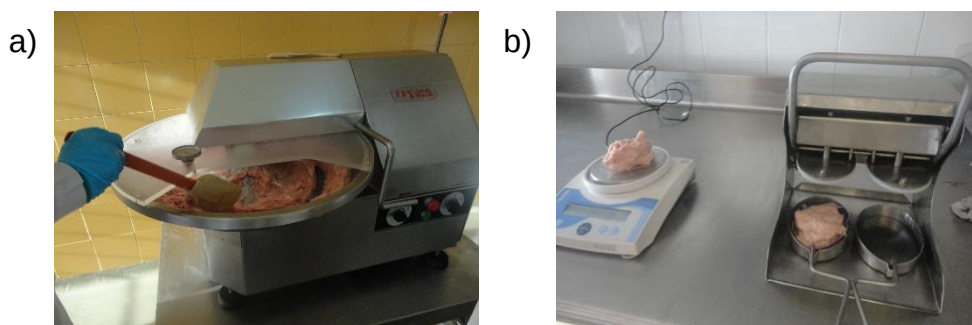
3.2 PROCESSAMENTO DOS EMULSIONADOS TIPO HAMBÚRGUER DE FRANGO

3.2.1 Experimento 1: Efeitos dos métodos de cozimento na oxidação lipídica e proteica de emulsionado tipo hambúrguer de frango pronto para consumo

Foram utilizadas coxas e sobrecoxas desossadas e resfriadas de frangos, adquiridas no dia do processamento, em um mercado local da cidade de Cáceres. Um total de 64 unidades do emulsionado tipo hambúrguer de frango foram preparadas em dois processamentos distintos, utilizando-se a seguinte formulação: 80% de carne, 2% de cloreto de sódio, e 18% de água gelada.

Para a preparação do emulsionado, as coxas e sobrecoxas de frango foram cortadas manualmente com auxílio de facas, homogeneizadas por 6 minutos, juntamente com o sal e água gelada, em cutter (Mainca®, EUA), seguido de moldagem (~ 100g) em forma de 10 cm de diâmetro e 1 centímetro de espessura (Figura 4). Amostras cruas (n=4) foram armazenadas a -80°C para serem analisadas posteriormente, com objetivo de caracterizar o produto.

Figura 4: Processamento dos emulsionados tipo hambúrguer de frango:



Após a etapa de moldagem os emulsionados foram submetidos a três processos distintos de cocção:

- i) Por imersão em água quente (80°C/30 minutos; n=20) utilizando um banho-maria (Ovan®) com controle automático de temperatura (Figura 5a). Para este processo de cocção, cada emulsionado foi embalado individualmente em saco plástico resistente ao calor e imerso na água previamente aquecida (80°C);

- ii) Por cozimento em forno (Unox®) (170°C/16 minutos; n = 20), onde após 8 minutos os emulsionados foram virados (Figura 5b).
- iii) Por cozimento em chapa (250°C/8 minutos; n = 20) utilizando-se chapa aquecedora (Repagas®) onde cada superfície dos emulsionados foi submetido a contato direto com a chapa por 4 minutos (Figura 5c);

Figura 5: Métodos de cocção aplicados nos emulsionados tipo hambúrguer de frango

a) Banho-maria



b) Forno



c) Chapa



Após o processo de cozimento, os emulsionados de frango (n=4 para cada processo de cocção) foram imediatamente congelados a -80°C para análises posteriores. Os demais emulsionados de frango cozidos (n=12 para cada processo de cozimento) foram dispostos em bandejas de polipropileno, envoltos com filme de policloreto de vinila (permeabilidade ao oxigênio: ~ 17 cm³/m² dia atm; permeabilidade da umidade: <5 g / m² dia; Tecnodur SL, Valencia, Espanha) e armazenado sob refrigeração a +4 °C por 14 dias.

Aos 7 e 14 dias de armazenamento refrigerado (+4°C) quatro unidades dos emulsionados (para cada processo de cozimento) foram congeladas em ultra-freezer a -80 °C para análises posteriores. Paralelamente, neste mesmo período, as quatro unidades restante (para cada processo de cozimento) foram reaquecidas em forno micro-ondas (600mW/1min) Marca Daewoo (Mod KOR-6L75; Daewoo Electronics Sales UK Ltd), seguido de congelamento a -80 °C para posteriores análises, por um período de até 30 dias.

3.2.2 Experimento 2: Efeito do uso de antioxidante natural e de embalagem com atmosfera modificada na oxidação lipídica e proteica de emulsionado tipo hambúrguer de frango pronto para consumo

O segundo experimento consistiu no processamento de duas formulações, uma controle e outra adicionada de extrato vegetal com potencial antioxidante conforme comprovado por López (2015). Um total de 56 emulsionados de frango foram preparados em duas bateladas para cada formulação seguindo o mesmo fluxo de processo e formulação (80% de carne, 2% de cloreto de sódio, 18% de água gelada) utilizados no experimento 1. Na formulação denominada de “com antioxidante” foi adicionado o extrato de *Quercus ilex* L., caracterizada conforme descrição do item 3.2.2.1. As amostras adicionadas do extrato vegetal foram elaboradas usando a mesma formulação, onde a quantidade de água destilada (18%) foi substituída por um volume equivalente de extrato de *Quercus ilex*, resultando em um conteúdo de compostos fenólicos totais de 200 mg equivalentes de ácido gálico (GAE) por kg de carne de frango. Esta concentração foi definida em estudos realizados previamente (LÓPEZ, 2015) e a obtenção deste extrato está descrita no item 3.2.2.2. Após moldagem os emulsionados (n=12x2=24 para cada formulação) foram submetidos ao cozimento em forno (Unox®) a 170°C/16 minutos, método definido a partir dos resultados do primeiro experimento. Paralelamente amostras cruas (n=4 para cada tratamento) foram armazenadas a -80°C para serem analisadas a fim de caracterizar os emulsionados de frango.

Após o cozimento, os emulsionados tipo hambúrguer de frango (n=4 para cada formulação) foram imediatamente congelados em ultra-freezer a -80°C para análises posteriores. O restante dos emulsionados cozidos foi submetido a dois sistemas de embalagem:

- a) Em atmosfera modificada (8% de O₂, 27% de CO₂ e 65% de N₂, n=16 para cada tratamento);
- b) Em atmosfera rica em oxigênio (ar sintético convencional com 21% de O₂, n=16 para cada tratamento).

Os emulsionados foram embalados individualmente em bandejas plásticas próprias para este fim, em equipamento de atmosfera modificada “Flow-Pack” (Figura 6) marca Ulma (Modelo PV350 LS; Hixemb). Após o processo de embalagem os emulsionados foram mantidos em refrigeração (+4°C) por 14 dias.

Figura 6: Sistema de embalagem “Flow-Pack”



Após o armazenamento refrigerado, os emulsionados tipo hambúrguer de frango, dos tratamentos controle ($n = 4 \times 2$ embalagens = 8) e com antioxidante ($n = 4 \times 2$ embalagens = 8) foram imediatamente congelados a -80°C para análises posteriores. Estas análises foram realizadas com o intuito de avaliar o desenvolvimento do processo oxidativo ocorrido durante o armazenamento refrigerado. Os emulsionados restantes dos tratamentos controle ($n = 4 \times 2$ embalagens = 8) e com antioxidante ($n = 4 \times 2$ embalagens = 8) foram reaquecidos em forno micro-ondas (600mW/1min) (Mod KOR-6L75; Daewoo Electronics Sales UK Ltd). Após o reaquecimento, as amostras foram congeladas a -80 °C para análises posteriores.

3.2.2.1 Caracterização das *Quercus ilex* L.

As amostras de bolota (*Quercus ilex* L. subsp. *Ballota* (Desf.) Samp.) foram coletadas na fase de maturação completa na região de Cáceres, Espanha (altitude = 450 m) durante o inverno de 2015. Depois de colhidas à mão, as amostras foram imediatamente transferidas para o laboratório, limpas e ordenadas para eliminar frutos danificados e enrugados. Após essa seleção as amostras foram processadas para

caracterização e extração dos compostos bioativos. A composição centesimal (umidade, lipídios, proteína bruta, cinzas) dos frutos selecionados foram analisados de acordo com métodos da AOAC (AOAC, 2000).

3.2.2.2 Extração e caracterização dos compostos bioativos das *Quercus ilex* L.

Para a preparação do extrato de *Quercus ilex* L., os frutos foram descascados, finamente moído (<1 milímetro) e liofilizado. Cem gramas do produto liofilizado foram homogeneizados por 3 minutos com 150 mL de acetona 60% (v/v) usando um Ultra Turrax a 7500 rpm. Os homogenatos foram centrifugados a 600 g por 3 min a 4°C utilizando uma centrífuga de Eppendorf 5810R (Eppendorf, Hamburgo, Alemanha). Os sobrenadantes foram recolhidos e filtrados em papel de filtro Whatman n.º 54. Do primeiro resíduo foi extraído mais uma vez os compostos bioativos seguindo o procedimento descrito previamente. Os dois sobrenadantes foram combinados e a extração do solvente (acetona) foi realizada utilizando evaporador rotativo a 500 mbar com temperatura menor que 30°C. O procedimento de extração (solvente de extração, concentração, condições, entre outros) foi otimizada de um estudo preliminar (López, 2015) para maximizar a concentração de compostos bioativos. Após evaporação da acetona o extrato de compostos bioativos foi acondicionado com 200 mL de água destilada e imediatamente transferido para o laboratório com objetivo de quantificar o teor de fenólicos totais (TFT), taninos condensados e atividade sequestradora de radicais.

3.3 ANÁLISES DE QUALIDADE FÍSICO-QUÍMICA DOS EMULSIONADOS TIPO HAMBÚRGUER DE FRANGO

- Composição química

A composição química dos emulsionados de frango foi determinada a partir das análises de umidade e proteínas realizadas segundo a metodologia da AOAC (2000) de acordo com as diretrizes dos procedimentos número 950.46 e 928.08, respectivamente. O método de Folch et al. (1959) foi utilizado para quantificar os lipídeos dos emulsionados.

- Medição da cor instrumental

As medições dos parâmetros de cor foram efetuadas segundo as recomendações da CIE (2004). Foram realizadas as leituras de luminosidade (L^*), cor vermelha (a^*) e amarela (b^*), em triplicata de pontos aleatórios sobre a superfície dos emulsionados tipo hambúrguer de frango, utilizando um colorímetro portátil Minolta CR-300 (Minolta Co., Ltd, Osaka, Japão). As medições destes parâmetros foram realizadas nos emulsionados de frango a temperatura ambiente (20°C) com iluminante D65 e 0° de ângulo de visão, antes do congelamento das amostras a -80°C para ambos os experimentos realizados. O equipamento foi previamente calibrado com uma placa branca fornecida pelo fabricante.

- Perda de peso por cozimento

A perda de peso no cozimento foi calculada a partir das diferenças no peso da amostra crua (A) e amostras cozidas (B). Onde, % Perda por Cozimento = $[(A-B)/A] \times 100$.

-Análise do perfil de textura

A análise de perfil de textura foi efetuada à temperatura ambiente, com um analisador de textura TA-XT2i (Stable Micro Systems, Surrey, Reino Unido). Quatro amostras cilíndricas de cada tratamento foram obtidas com um cortador cilíndrico (2,5 cm de diâmetro e altura de 1,0 cm, aproximadamente). O cortador foi colocado perpendicularmente na superfície do emulsionado tipo hambúrguer e foi pressionado para baixo de modo que toda a lâmina de corte atravessasse a amostra e, em seguida, levantou-se para remover a amostra. As amostras foram sujeitas a um teste de compressão de dois ciclos. As análises foram realizadas em triplicata em cada amostra. As amostras foram comprimidas a 40% da sua altura original com uma sonda cilíndrica de 5 cm de diâmetro a uma velocidade de 5 mm/s. Os seguintes parâmetros foram determinados de acordo com (BOURNE, 1982): Dureza (N), a adesividade ($N \times s$), mastigabilidade (N), elasticidade (adimensional), coesividade (adimensional), e a resiliência (sem dimensão). Embora todos os parâmetros mencionados tenham sido determinados, apenas os resultados de dureza são mostrados devido sua elevada importância para o produto analisado.

- Análises dos compostos bioativos

O TFT foi determinado seguindo o método de Folin-Ciocalteu descrito por Singleton e Rossi (1965). Onde 25µL do extrato foi homogeneizado, em vórtex, com 175µL de água destilada, 1mL do reagente de Folin-Ciocalteu 10%, e 800µL de carbonato de sódio 7,5%. As amostras foram deixadas em repouso no escuro por 30 minutos e realizou-se a leitura das absorbâncias em espectrofotômetro a 765nm. A concentração de compostos fenólicos foi calculada utilizando-se uma curva padrão de ácido gálico. A quantidade total de compostos fenólicos foi expressa em miligramas de equivalentes de ácido gálico (GAE) por 100g de amostra.

Os taninos condensados foram determinados pelo procedimento butanol-HCl descrito por Porter et al. (1986) na polpa da *Quercus ilex*. Onde 50mg da polpa de *Quercus ilex* foi homogeneizada com 12 mL do reativo de Porter (Butanol/acetona-HCl 95:5 v/v, reativo férrico – 2% de sulfato amônico férrico em HCl 2N). Após a homogeneização as amostras foram incubadas em estufa a 70°C por duas horas, com agitação a cada 30 minutos. Após resfriamento das amostras e centrifugação a 2000rpm por 4 minutos, foi medida a absorbância do sobrenadante a 550nm. A quantificação dos taninos condensados foi realizada a partir de uma curva padrão de procianidina B1. Os resultados foram expressos em mg equivalentes de procianidina B1/100g de fruto.

A atividade sequestradora de radicais foi medida a partir do ensaio de 2,2-difenil-1-picrilhidrazil (DPPH) relatada por Kähkönen, Heinonen (2003). 10µL do extrato vegetal de *Quercus ilex* foi agitado com 2mL do radical livre DPPH 0,1mM em metanol. Após repouso, no escuro, por 6 minutos foram realizadas as leituras da absorbância a 517nm. A atividade antioxidante foi calculada a partir de curva padrão de Trolox, e os resultados foram expressos em porcentagem de inibição.

3.4 ANÁLISES DE OXIDAÇÃO LIPÍDICA

- Composição de ácidos graxos

Os metil-ésteres de ácido graxos foram preparados a partir da metilação a frio com solução metanólica de hidróxido de potássio (2N) segundo a metodologia de Cert,

Moreda, & Pérez-Camino, (2000). Eles foram separados usando o cromatógrafo a gás Hewlett–Packard HP-5890A, equipado com detector de ionização de chama e coluna capilar Supelco® (50m x 0,32mm de diâmetro interno; 1,0 µm). Os fluxos de gases foram 35 mL.min⁻¹ para o hidrogênio, 15 mL.min⁻¹ para o hélio e 400 mL.min⁻¹ para o ar sintético. O gás de arraste utilizado foi o hélio com fluxo de 1,7 mL.min⁻¹. A temperatura inicial da coluna foi programada para 180 °C durante 5 minutos, e foi aumentada para 210°C a 1°C min⁻¹. Subsequentemente, a temperatura foi aumentada para 250°C a 4°C min⁻¹ e mantida na temperatura máxima por 10 minutos, resultando num tempo total de corrida de 55 minutos. Uma mistura de 37 ésteres metílicos de ácidos graxos da Supelco® (Sigma-Aldrich, EUA) foi utilizada como padrão de referência para identificar os picos de ácidos graxos.

- Medição das substâncias reativas ao ácido tiobarbitúrico (TBARS)

As substâncias que reagem ao ácido tiobarbitúrico (TBARS) foram extraídas em condições ácidas e quantificadas espectrofotometricamente seguindo o método descrito por Estévez & Cava (2004). Onde 2,5 g de amostra foram homogeneizadas em turrax, mantidas em condições frias, com ácido perclórico (3,86%) e BHT (4,2%). Após centrifugação (2000 g por 4 minutos) e filtração em papel de filtro Whatman n°1, o sobrenadante foi aferido em balão de 10 mL com ácido perclórico (3,86%). A 2 mL deste filtrado foi adicionado 2 mL de TBA 0,02M, em tubos de ensaio com tampa rosqueada, que foram mantidos a 90°C por 30 minutos. Após resfriamento e leitura das absorbâncias a 532 nm, os valores de malonaldeído (MDA) (mg de MDA/kg de amostra) foram calculados com auxílio de um branco (contendo ácido perclórico e TBA) e uma curva padrão de Tetraetoxipropano (TEP) que variou de $5,7 \times 10^{-5}$ a $1,1 \times 10^{-3}$ mg TEP.mL⁻¹.

- Determinação dos compostos de Maillard

Para a extração dos compostos coloridos formados após o pré-cozimento (compostos de Maillard) foi utilizado o seguinte protocolo adaptado de Ramirez et al (2004): cerca de 0,5 g de amostra foi finamente triturado e colocado num tubo de ensaio com tampa. Uma mistura de metanol:água MiliQ (1: 1, v/v) foi utilizado para a extração dos compostos coloridos. Os tubos foram hermeticamente fechados e colocados em agitador orbital por 1 h a temperatura ambiente. Após centrifugação a

5000 g por 20 min, as absorbâncias dos sobrenadantes foram lidas a 420 nm. O coeficiente de extinção molar de melanoidinas ($1,00 \pm 0,03 \text{ L mmol}^{-1} \text{ cm}^{-1}$) relatado por Martins & Van Boekel, (2003) foi aplicada para calcular a concentração de produtos coloridos nas amostras.

3.5 ANÁLISES DA OXIDAÇÃO PROTEICA

- *Quantificação dos compostos carbonílicos*

A oxidação das proteínas foi avaliada a partir da quantificação de compostos carbonílicos totais das amostras após derivatização com 2,4 dinitrofenilhidrazina (DNPH) segundo a metodologia de Ganhão, Morcuende, & Estévez, (2010) com algumas modificações. Cerca de 1 g de amostra foi homogeneizada com tampão Na_3PO_4 20mM e NaCl 0,6M com pH 6,5 (1:10) (p/v). Deste homogeneizado, 150 μL foram utilizados para determinar a concentração de proteínas e o conteúdo de compostos carbonílicos. Em ambos os casos, as proteínas foram precipitadas com 1mL de ácido tricloroacético (TCA) a 10% após centrifugação a frio ($+4^\circ\text{C}$) a 2400g. Para a determinação dos compostos carbonílicos foi adicionado 1 mL de HCl 2M e DNPH 0,2%. Para a determinação das proteínas foi adicionado apenas 1 mL de HCl 2M. Após incubação a temperatura ambiente por 1 h as proteínas foram precipitadas novamente com 1 mL de TCA 10% e centrifugação a 9000 g por 10 min. Após duas lavagens com 1 mL de etanol/acetato de etila (1:1 v/v) seguidas de centrifugações a 9000g por 5 min, entre cada lavagem as proteínas precipitadas foram dissolvidas em 1,5 mL de tampão Na_3PO_4 20mM; pH 6,5 adicionado de cloridrato de guanidina 6M. A concentração de proteínas das amostras foi calculada a partir da leitura das absorbâncias a 280 nm utilizando uma curva padrão de albumina sérica bovina (BSA). A quantidade de carbonílicos foi expressa em nmoles de carbonílicos por mg de proteína, usando um coeficiente de extinção molar de hidrazonas ($21,0 \text{ nM}^{-1} \text{ cm}^{-1}$) com leituras da absorbância a 370nm.

- *Determinação de tióis livres e ligações dissulfeto*

Os tióis livres e as ligações dissulfeto foram analisados seguindo a metodologia de Rysman et al., (2014), onde 1 g de amostra foi homogeneizado em ultraturrax com 25 mL de tampão tris 100mM; pH 8,0 adicionado de hidrocloreto de Guanidina

(GuHCl) 6M. Após centrifugação (5400g por 20 min a +4°C) e filtração (papel Whatman nº1) do sobrenadante, foi determinada a concentração de proteínas espectrofotometricamente a 280nm usando curva padrão de BSA. As amostras e os filtrados foram mantidos em gelo durante toda a análise. Uma alíquota de 3mL do filtrado foi submetido a redução de dissulfeto por adição de 50 µL de 1-octanol (como um agente anti-espuma) e 100 mL boro-hidreto de sódio (30% w/v) em NaOH 1M, preparado na hora da análise. Após incubação a 50 °C por 30 min, 1,35 mL de HCl 6 M foi adicionado, e mantido em agitação por 10 min. Os tióis livres e totais foram determinados com 4-4'-Dithiodipiridina (4-DPS) nos filtrados não-reduzidos e reduzidos, respectivamente. Uma alíquota de 500 µL do filtrado foi misturada com 2 mL de tampão ácido cítrico a 1M; pH 4,5 adicionado de GuHCl 6 M e 500 µL da solução de 4-DPS (4-DPS 4 mM em HCl 12 mM). A absorbância foi medida a 324 nm contra o tampão de ácido cítrico 1M pH 4,5 adicionado de GuHCl 6M antes da adição de 4-DPS (A_{pre}) e após 30 minutos de reação com 4-DPS no abrigo da luz e a temperatura ambiente (A_{pos}). Uma mistura de 2,5 mL de tampão de ácido cítrico 1 M pH 4,5 adicionado de GuHCl 6M e 500 µL da solução de 4-DPS foi preparada como um branco (A_{branco}). A absorbância correspondente a concentração de tiol foi calculada subtraindo A_{pre} e A_{branco} de A_{pos} .

A concentração de tiol foi calculada a partir de uma curva padrão (2,5 a 500 µM) de L-cisteína em tampão de ácido cítrico 1 M pH 4,5 adicionado de GuHCl 6M. O conteúdo de tiol foi expresso como nanomoles de tiol por miligrama de proteína. Como o tratamento de ligações dissulfeto com boro-hidreto de sódio produz dois grupos tiol, o teor de ligações dissulfeto foi calculada como metade da diferença entre os tióis total e livres.

- Medição da Bases de Schiff e Triptofano

As medições dos compostos fluorescentes (triptofano e base de Schiff) foram medidas a partir de espectroscopia de fluorescência segundo a metodologia de Estévez, et al. (2008) com algumas modificações. As amostras dos emulsionados de frango foram homogeneizadas (1:5 p/v) em tampão fosfato de sódio pH 6,0 adicionado de uréia 8M com auxílio de ultraturrax por 1 min. O volume de 1,5 mL de tampão fosfato de sódio 20mM; pH 6,5 com cloridrato de guanidina 6M foram adicionado a 0,5 mL do homogeneizado e agitado em vortex. O extrato aquoso foi transferido para uma

cubeta de quartzo com as quatro paredes lisas de 4 mL (Hellma Analytics, 101-QS 10x10mm). O espectro de emissão para a base de Schiff foi registrado entre 400 e 500 nm de comprimento de onda com excitação a 350 nm, enquanto para o triptofano o espectro de emissão foi registrado entre 300 e 400 nm de comprimento de onda com excitação a 283nm (LS 55 Perkin-Elmer espectrômetro de luminescência, Beaconsfield, Reino Unido). A excitação e emissão de larguras de fenda foram fixado em 10 nm e os dados foram coletados em 500 nm por min. Os resultados foram expressos como unidades de intensidade de fluorescência emitida por proteína dos produtos de oxidação a 450 nm. Estes valores foram corrigidos de acordo com o teor de proteína de cada amostra multiplicando pelo fator de correção Cf ($Cf = Pt/Pp$), onde Pt é a média total dos teores de proteína de todas as amostras e Pp é a média do teor de proteína de cada tratamento analisado.

- *Determinação da digestibilidade 'in vitro'*

A digestibilidade das proteínas dos emulsionados de frango foi determinada *in vitro* por uma exposição sequencial de proteases digestivas seguindo o procedimento descrito por Santé-Lhotellier et al. (2008) com algumas modificações. As amostras foram homogeneizadas em tampão fosfato 100 mM com NaCl 0,6M pH 6,5 (1:10, p/v). As proteínas foram precipitadas primeiro por TCA a 10% e centrifugação (5000 g, 5 min., + 4 °C) e, em seguida, lavadas em tampão de glicina 33 mM a pH 1,8 (o pH gástrico). As proteínas (concentração ajustada a 1 mg/mL) foram digeridas primeiro com pepsina da mucosa gástrica suína (20 U/mg de proteína) por 1 h a 37 °C. A digestão foi terminada com a adição a vários tempos (0, 10, 20, 30, 40, 60 min.) de 15% (concentração final) de TCA e centrifugação subsequente a 5000 g por 5 minutos. O teor de peptídeos/aminoácidos hidrolisados no sobrenadante foi calculada a partir da absorbância a 280 nm e a velocidade de proteólise foi expressa em unidades de densidade óptica por hora ($\Delta OD/hora$).

Os precipitados de proteína obtidos após centrifugação do hidrolisado de pepsina por 60 min foram lavados duas vezes em tampão de glicina 33 mM a pH 8 (pH duodenal) e a concentração de proteína foi calculada no mesmo tampão. As proteínas foram em seguida hidrolisadas por uma mistura de tripsina e α -quimotripsina do pâncreas de suínos por 30 min a 37 °C (6,6 L e 0,33 U/mg de proteína, respectivamente). A digestão foi terminada com a adição a vários tempos (0, 5, 10,

20, 30 min.) de 15% (concentração final) de TCA e a taxa de proteólise foi determinada como descrito anteriormente.

3.6 ANÁLISE DE COMPOSTOS VOLÁTEIS

Os compostos voláteis foram analisados nas amostras cozidas, refrigeradas e reaquecidas (dos experimentos 1 e 2) pela técnica de headspace-SPME em cromatógrafo a gás Hewlett-Packard 5890 série II, acoplado a um detector seletivo de massa Hewlett-Packard HHP-5791A, seguindo o método desenvolvido por Estévez et al. (2003). Um grama de amostra triturada foi pesado num frasco de 4 mL e foram fechados com septo de teflon/silicone (National Scientific). Uma fibra de SPME (Revestimento 50/30 μm divinilbenzeno-carboxen-polidimetilsiloxano), pré-condicionada previamente a análise (220 °C por 45 min), foi inserida por meio do septo e exposta ao espaço superior do frasco por 30 min. Antes da inserção e exposição da fibra os frascos contendo as amostras, foram aquecidos por 10 min a 37 °C em forno estufa. Após o tempo de exposição, a fibra de SPME foi imediatamente transferida para o injetor do cromatógrafo que estava no modo de *splitless*. A separação dos compostos voláteis foi realizada em uma coluna capilar de fase estacionária de sílica fundida com 5% fenil-metil de silicone (HHP-5) (Hewlett-Packard, 50 m x 0,32 mm ID, espessura do filme 1,05 μm). O gás de arraste foi hélio a 18,5 psi resultando num fluxo de 1,6 mL.min⁻¹ a 40 °C. A fibra de SPME foi dessorvida e mantida no injetor a 220 °C durante todo o tempo de corrida da cromatografia. O programa da temperatura foi isotérmico por 10 min a 40 °C e, em seguida, aumentada a uma taxa de 7 °C.min⁻¹ até 250 °C e mantida durante 5 minutos. A temperatura da linha de transferência do GC-MS foi de 270 °C. O espectrômetro de massa operou no modo de impacto de elétrons com uma energia eletrônica de 70 eV e um multiplicador de tensão de 1650 V e os dados foram recolhidos a uma taxa de 1 leitura s⁻¹ ao longo de uma gama de m/z 40-300. Os compostos foram identificados positivamente por comparação de seus espectros de massa com compostos padrão (Sigma-Aldrich, Steinheim, Alemanha).

3.7 ANÁLISE SENSORIAL

A avaliação da aceitação odor dos hambúrgueres de frango prontos para consumo foi realizada por 30 e 50 pessoas (funcionários e estudantes da Faculdade de Veterinária, em Cáceres-Espanha) para o primeiro experimento e para o segundo experimento, respectivamente. Foi utilizada escala hedônica de sete pontos variando de extremamente desgostei (1) a extremamente gostei (7). Cinco gramas de cada amostra foram finamente cortados e dispostas em tubos fechados e embrulhados com papel alumínio. As amostras foram oferecidas aos provadores, após aquecida a 37 °C por 10 min em forno estufa. Cada amostra foi codificada com três números aleatórios.

Para a aceitação da cor, cilindros de hambúrgueres com dimensões de 2,5 cm de diâmetro e 1,0 cm de altura, aproximadamente, foram oferecidos aos provadores para avaliação visual. Também foi utilizada escala hedônica de sete pontos variando de extremamente desgostei (1) a extremamente gostei (7). Cada amostra foi codificada com três números aleatórios.

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4 RESULTADOS

Os resultados dos experimentos realizados estão apresentados em forma de artigos científicos publicados e submetidos em periódicos de impacto na área de Ciência e Tecnologia de Alimentos. São eles:

Artigo I: Effect of pre-cooking methods on the chemical and sensory deterioration of ready-to-eat chicken patties during chilled storage and microwave reheating
(Publicado no periódico Journal of Food Science and Technology, em junho de 2016)

Artigo II: Oxidative degradation of essential amino acids and impaired in vitro protein digestibility in ready-to-eat (RTE) chicken patties
(Submetido ao periódico Food Chemistry, em: 08 de fevereiro de 2015 - Anexo A)

Artigo III: Phytochemicals from acorn (*Quercus ilex* L.) inhibit oxidation and enhance consumer liking of ready-to-eat chicken patties irrespective of packaging atmosphere
(Submetido ao periódico Food Chemistry, em: 16 de junho de 2016- Anexo B)

Effect of pre-cooking methods on the chemical and sensory deterioration of ready-to-eat chicken patties during chilled storage and microwave reheating

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Abstract The effects of pre-cooking methods, namely, boiling (BL), roasting (RT) and grilling (GR), refrigerated storage (14 days/+4 °C) and microwave reheating on chicken patties were studied. Physical, chemical and sensory parameters were evaluated in order to correlate the chemical deterioration of ready-to-eat chicken patties with the acceptance of the odor. Chemical deterioration was evaluated through the chemical composition, Maillard compounds, Thiobarbituric acid-reactive substances (TBARS) and volatiles. Sensory deterioration (odor liking) was performed by an acceptance test with hedonic scale. According to the TBARS values and volatile compounds generated in the head space during the examined stages, the pre-cooking method and the storage time had a significant effect on lipid oxidation, whereas reheating in a microwave had a negligible impact. At each succeeding processing stage, panelists gave lower odor scores to all samples and no significant differences were found between treatments at any stage. RT and GR patties showed less intense chemical changes and presented higher acceptance scores by the sensory panel than BL patties. Thus, the choice of pre-cooking method and control of storage conditions plays a key role in the inhibition of oxidative changes in ready-to-eat chicken patties.

Keywords Convenience food · Pre-cooking · Microwave reheating · Lipid oxidation · Odor

Introduction

Consumers appreciate ready-to-eat (RTE) food items for being (i) a convenience way to save time and effort, (ii) a good alternative to other fast food and (iii) a solution to a growing lack of cooking skills (Olsen et al. 2009). On this line, Europe has witnessed in the last 5 years an increase in the consumption of heat-and-eat and RTE meat and poultry, such as meat balls, burger patties, sausages and other processed muscle foods (Analytics 2014). The market of convenience food in Europe was calculated to have a value of 47 bill euros in 2012, almost 20 % higher than in 2007 (Kanzler and Wagner 2009).

The shift from freshly prepared dishes to processed RTE food may involve a loss of quality in terms of sensory properties and nutritional value. The manufacture of these items typically involves a number of consecutive processing stages, namely, pre-cooking, cold storage and a final reheating step prior to consumption (Kanzler and Wagner 2009). The long and severe technological processes applied to the meat, which includes size reduction, salting, and various heating steps, facilitates the onset of oxidative reactions that affect, in turn, the sensory properties and nutritional value of the final meat product (Pegg and Shahidi 2012; Soladoye et al. 2015). In particular, cooked meats are known to develop a fast and intense flavor deterioration during the subsequent cold storage commonly described as warmed-over-flavor (WOF) (Pegg and Shahidi 2012). This deterioration process is particularly relevant in poultry products owing to their high degree of fat unsaturation and high susceptibility to oxidative reactions (Pegg

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and Shahidi 2012). While the onset of lipid oxidation and WOF has been profusely studied in cooked and subsequently refrigerated meat in terms of influential factors (Byrne et al. 2001a, b), methodological approach (Brøndum et al. 2000; Sullivan et al. 2003) and sensory characterization of WOF (Byrne et al. 1999), the evolution of lipid oxidation and key-odorants during the whole processing (including the final heating stage) requires an in-depth consideration. Some studies have examined the effect of assorted cooking procedures (frying, boiling, roasting, among others) on the chemical composition and quality traits on muscle foods (Estévez et al. 2003; Ramírez et al. 2005; Roldán et al. 2014a, b). The nature of the cooking method determines the mass and energy transfer phenomena, the modification of the meat and hence, the properties of the cooked product (Ramírez et al. 2005; Matsuda et al. 2013; Roldán et al. 2014a, b). However, there is little information in the scientific literature on the impact of the pre-cooking method on the susceptibility of a convenience meat product to suffer further deterioration during the following processing stages (chilled storage and reheating).

Therefore, the aim of the present study was to investigate the effect of assorted pre-cooking methods (boiling, roasting and grilling) on the physico-chemical properties of chicken patties and on their susceptibility to undergo lipid oxidation and odor deterioration during the succeeding processing stages: refrigerated storage and final reheating.

Materials and methods

Chemicals and reagents

All chemicals were supplied from Panreac (Panreac Quimica, S.A., Barcelona, Spain), Merck (Merck, Darmstadt, Germany), Sigma Chemicals (Sigma-Aldrich, Steinheim, Germany) and Scharlau (Gillman, South Australia). Water used was purified by passage through a Milli-Q system (Millipore Corp., Bedford, MA, USA).

Processing of chicken patties

The chicken patties were made with chicken thigh and drumstick (1:1, w/w) obtained from a local market in the city of Cáceres (Spain). A total of 64 chicken patties were prepared in two separate processes following the general basic recipe: 80 % meat, 2 % sodium chloride, and 18 % of cold water. Following the procedure described by Ganhão et al. (2010), emulsified experimental patties were produced to guarantee a homogeneous product. After 6 min of homogenization in cutter (Mainca®, USA) the chicken patties (~100 g) were molded in a burgermarker

(10 cm diameter and 1 cm thickness) and submitted to three processes of pre-cooking: boiling (80 °C/30 min) (n = 20); grilling (250 °C/8 min) (n = 20); and roasting (170 °C/16 min) (n = 20). All temperatures refer to internal temperatures measured during cooking in the core of patties using a digital probe thermometer (Testo thermocouple, Mod. 735-1, Lenzkirch, Germany). Raw samples (prior to pre-cooking) were also analyzed in order to characterize the chicken patties. Boiled patties were packed in a plastic bag and immersed in a thermostated water bath. Grilling was made on an electrical griddle (Repagas, 550 series, Madrid, Spain) while roasting was performed in an electrical oven (Unox®, Mod. GN2.1, Cadoneghe, Italy). Each pre-cooking method was also performed twice. One set of pre-cooked patties (n = 4 for each pre-cooking method) was immediately frozen at −80 °C for further analyses. The remaining pre-cooked patties were dispensed in polypropylene trays, wrapped with polyvinyl chloride film (oxygen permeability: ~17 cm³/m² day atm; moisture permeability: <5 g/m² day; Tecnodur S.L., Valencia, Spain) and stored at +4 °C for 14 days. Samplings (n = 8 for each pre-cooking method) were performed at days 7 and 14. Half of the patties (n = 4 for each pre-cooking method and sampling time) were frozen at −80 °C for further analyses. The other half of pre-cooked and refrigerated patties (n = 4 for each pre-cooking method and sampling time) were reheated in a microwave (600 mW/1 min; Daewoo, Mod. KOR-6L75; Daewoo Electronics Sales UK Ltd). Upon reheating, samples were also frozen at −80 °C for further analyses.

Analytical methods

Chemical composition and fatty acid profiles

Moisture and total protein were determined using AOAC methods (AOAC 2000). The method of Folch et al. (1957) was used for determining fat content in patties. Fatty acid methyl esters (FAMES) were prepared by methylation with cold a methanolic solution of potassium hydroxide (Cert et al. 2000). FAMES were separated using a Hewlett-Packard HP-5890A gas chromatograph, equipped with an on-column injector, a flame ionization detector and a capillary column Supelco® (50 m × 0.32 mm internal diameter, 1.0 µm film). Detector gas flow rates were 35 mL min^{−1} for the hydrogen, 15 mL min^{−1} for the helium and 400 mL min^{−1} for the synthetic air. Helium was used as the carrier gas (flow rate 1.7 mL min^{−1}). The initial column temperature was programmed to 180 °C for 5 min, which was then increased to 210 °C at 1 °C min^{−1} and held at the maximum temperature for 30 min. Subsequently, the temperature was increased to 250 °C at 4 °C min^{−1} and held at the maximum temperature for 20 min,

resulting in a total run time of 55 min. The Supelco 37 Component FAME Mix (Sigma-Aldrich, Steinheim, Germany) was used as a reference standard to identify the fatty acid peaks.

Cooking loss

The cooking loss was calculated from differences in the weight of raw (W_R) and cooked samples (W_C) as follows:
 $\% \text{ Cooking Loss} = ((W_R - W_C)/W_R) \times 100$.

Maillard reaction products (MRPs) quantification

For extraction of colored materials formed by cooking, 0.5 g of each sample was finely ground and placed in a glass tube. A mixture of methanol:water milliQ (1:1, v/v) was used for the extraction of colored materials. Tubes were hermetically closed and placed in an orbital shaker. After 1 h, tubes were centrifuged at 5000g for 20 min. Pellets were discarded and the absorbances of supernatants were recorded at 420 nm. The molar extinction coefficient ($1.00 \pm 0.03 \text{ l mmol}^{-1} \text{ cm}^{-1}$) reported by Martins and Van Boekel (2003) was applied to calculate the concentration of MRPs in our samples.

Thiobarbituric acid-reactive substances (TBARS)

Thiobarbituric acid-reactive substances (TBARS) were extracted in acidic conditions and quantified spectrophotometrically following the method described by Ganhão et al. (2011).

Analysis of volatiles

The odor-active volatiles was analyzed in pre-cooked, chilled and reheated samples by headspace-SPME and GC/MS (gas chromatograph Hewlett–Packard 5890 series II coupled to a mass selective detector Hewlett–Packard HHP-5791A) following the method reported by Estévez et al. (2003). 1 gram of minced sample was weighed into a 4 mL vial. An SPME fibre (50/30 μm divinylbenzene–carboxen–polydimethylsiloxane coating) was inserted through the septum and exposed to the headspace of the vial. The SPME fiber was preconditioned prior analysis at 220 °C during 45 min. Vials were preconditioned for 10 min at 37 °C. Extraction was carried out at 37 °C for 30 min in an oven. After extraction, the SPME fiber was immediately transferred to the injector of the chromatograph which was in splitless mode. The separation of volatile compounds was performed on a 5 % phenylmethyl silicone (HHP-5)-bonded-phase fused-silica capillary column (Hewlett–Packard, 50 m $\times$ 0.32 mm i.d., film

thickness 1.05 m). The carrier gas was helium at 18.5 psi, resulting in a flow of 1.6 mL min^{-1} at 40 °C. The SPME fiber was desorbed and maintained in the injection port at 220 °C during the whole chromatography run. The temperature program was isothermal for 10 min at 40 °C and then increased at the rate of $7 \text{ }^\circ\text{C min}^{-1}$ to 250 °C and held for 5 min. The GC–MS transfer line temperature was 270 °C. The mass spectrometer operated in the electron impact mode with an electron energy of 70 eV and a multiplier voltage of 1650 V and collected data at a rate of 1 scan s⁻¹ over a range of m/z 40–300. Compounds were positively identified by comparing their mass spectra with those from standard compounds (Sigma-Aldrich, Steinheim, Germany). Results are expressed as area units (AU) $\times 10^6$.

Objective color measurement

Color measurements were performed by measuring lightness (L^*), redness (a^*) and yellowness (b^*) values in triplicate at different random points on the surface of the chicken patties using a portable Minolta Chromameter CR-300 (Minolta Co., Ltd, Osaka, Japan) equipped with a measuring head (CR-300). Color measurements were made on patties at ambient temperature ($\sim 20 \text{ }^\circ\text{C}$) with an illuminant D65 and a 0° angle observer prior to frozen storage at $-80 \text{ }^\circ\text{C}$. The equipment was previously calibrated with the white tile provided by the supplier.

Odor liking assessment

The odor liking assessment of ready-to-eat chicken patties was performed by 30 untrained panelists (staff and students from the Veterinary Faculty in Caceres, Spain). Panelists were regular consumers of processed muscle foods and ranged in age from 25 to 60. A hedonic scale of seven points from extremely disliked (1) to extremely liked (7) was used. Five grams of each sample were finely minced, dispensed in falcon tubes, sealed and wrapped with aluminum foil and offered to the panelists after being warmed up to 37 °C for 10 min in oven. Each sample was coded with three random numbers.

Statistical analysis

Four ready-to-eat chicken patties per treatment and sampling point were prepared in corresponding replicated processes and used as experimental units. The effect of the technological treatments on each measured parameter was analyzed by one-way analyses of variance (ANOVA) and Tukey's tests. A Kruskal–Wallis analysis was applied to sensory data. A significance level of $p < 0.05$ was considered.

Results and discussion

Impact of pre-cooking method on composition of chicken patties

The chemical composition of the chicken patties is shown in Table 1. After pre-cooking, all treatments had lower moisture values than raw samples owing to the dehydration of samples during the heat treatment. Roasted (RT) samples presented the lowest moisture value (67.66 %), the boiled (BL) treatment led to samples with the highest moisture content (71.33 %) while grilled (GR) samples had intermediate moisture values (68.82 %). Regarding to lipid content, no differences were observed ($p > 0.05$) between BL and raw samples, which had 9.08 and 9.93 %, respectively. However, RT and GR patties had 1.09 and 1.18 times higher lipid contents than raw samples, respectively. These results, as well as the highest protein value in RT samples, may be attributed to a highest loss of water during pre-cooking (Table 1). The fatty acid composition was not influenced by the pre-cooking method ($p > 0.05$). Chemical composition of the present pre-cooked chicken patties is comparable with similar cooked poultry products characterized in previous studies (Conchillo et al. 2005; Mora et al. 2011). Mass and energy

transfer processes, which occur differently in each pre-cooking method, can be mainly responsible for the observed behaviors (Adedeji et al. 2009).

Impact of pre-cooking method on cooking loss, Maillard compounds and color of chicken patties

The cooking loss values, color parameters (L^* , a^* and b^*) and Maillard compounds are shown in Table 2. The losses found in RT (17.31 %) and GR samples (13.15 %) were consistent with those observed by Domínguez et al. (2014) who studied the influence of these cooking methods in foal meat. The BL treatment caused the lowest cooking loss (4.51 %), a fact confirmed by Juárez et al. (2010) when analyzing the influence of different cooking methods on buffalo meat. The low values observed for BL treatment may be due to (i) the mild temperatures applied during cooking and (ii) the hindrance of water evaporation during cooking as the samples were packed in plastic bags through which the heat transfer occurred (Kanzler and Wagner 2009).

Regarding the color after cooking, the RT and GR chicken patties were, in general, darker, less red and more yellow than raw samples (Table 2). BL samples, conversely, were as yellow as the raw samples and displayed a

Table 1 General and fatty acid composition (mean $\pm$ SD) of chicken patties subjected to different pre-cooking methods

	R	Boiled	Roasted	Grilled	p^a
Moisture ^b	72.78a $\pm$ 0.20	71.33b $\pm$ 0.65	67.66d $\pm$ 0.18	68.82c $\pm$ 0.50	<0.001
Protein ^b	14.25c $\pm$ 0.33	16.10b $\pm$ 0.57	17.33a $\pm$ 0.53	16.18b $\pm$ 0.54	<0.001
Lipid ^b	9.93bc $\pm$ 0.70	9.08c $\pm$ 0.46	10.87ab $\pm$ 0.60	11.71a $\pm$ 0.73	<0.001
Fatty acid profile ^c					
C12	0.06 $\pm$ 0.02	0.07 $\pm$ 0.01	0.06 $\pm$ 0.01	0.06 $\pm$ 0.01	0.794
C14	1.23 $\pm$ 0.05	1.19 $\pm$ 0.05	1.22 $\pm$ 0.07	1.23 $\pm$ 0.09	0.797
C16	29.73 $\pm$ 0.50	29.04 $\pm$ 0.61	29.63 $\pm$ 1.58	29.37 $\pm$ 2.11	0.893
C16:1	8.76 $\pm$ 0.33	8.74 $\pm$ 0.45	8.45 $\pm$ 0.54	8.78 $\pm$ 0.50	0.712
C17	0.14 $\pm$ 0.02	0.15 $\pm$ 0.01	0.14 $\pm$ 0.02	0.15 $\pm$ 0.02	0.750
C17:1	0.09 $\pm$ 0.03	0.12 $\pm$ 0.02	0.12 $\pm$ 0.01	0.11 $\pm$ 0.01	0.253
C18	5.32 $\pm$ 0.14	5.26 $\pm$ 0.12	5.35 $\pm$ 0.53	5.23 $\pm$ 0.48	0.963
C18:1n-9	40.46 $\pm$ 1.31	40.33 $\pm$ 1.03	40.00 $\pm$ 1.89	40.06 $\pm$ 2.78	0.983
C18:2n-6	12.36 $\pm$ 1.35	13.19 $\pm$ 1.44	13.09 $\pm$ 1.31	13.10 $\pm$ 0.66	0.764
C18:3n-6	0.11 $\pm$ 0.01	0.11 $\pm$ 0.01	0.11 $\pm$ 0.01	0.11 $\pm$ 0.01	0.757
C18:3n-3	0.99 $\pm$ 0.15	1.07 $\pm$ 0.14	1.07 $\pm$ 0.14	1.06 $\pm$ 0.08	0.779
C20:1	0.26 $\pm$ 0.01	0.25 $\pm$ 0.02	0.26 $\pm$ 0.02	0.26 $\pm$ 0.02	0.939
C20:2	0.10 $\pm$ 0.02	0.10 $\pm$ 0.01	0.11 $\pm$ 0.01	0.10 $\pm$ 0.02	0.569
C20:4n-6	0.38 $\pm$ 0.03	0.38 $\pm$ 0.01	0.39 $\pm$ 0.04	0.39 $\pm$ 0.04	0.964

Different letters denote significant differences ($p < 0.05$) between pre-cooking methods

R Raw patties

^a Statistical significance in ANOVA

^b Data expressed as g/100 g sample

^c Data expressed as percentage of total fatty acids analyzed

Table 2 Cooking loss, instrumental color and Maillard reaction products (MRPs), in chicken patties subjected to different pre-cooking methods

	R	Boiled	Roasted	Grilled	<i>p</i> ^a
Cooking loss ^b	–	4.51c ± 0.63	17.31a ± 2.18	13.15b ± 1.91	<0.001
L*	73.03b ± 0.48	74.49a ± 1.24	65.65d ± 1.90	67.61c ± 1.35	<0.001
a*	11.74a ± 0.43	4.34b ± 0.39	4.81b ± 0.93	4.44b ± 0.83	<0.001
b*	11.99c ± 0.30	11.68c ± 0.42	17.90b ± 0.77	22.61a ± 3.70	<0.001
Chroma	16.79b ± 0.41	12.46c ± 0.49	18.55b ± 0.78	23.07a ± 3.62	<0.001
Hue	44.41a ± 1.12	20.37b ± 1.41	15.04c ± 2.82	11.38d ± 2.86	<0.001
MRPs ^a	–	15.91c ± 2.49	30.91b ± 7.69	45.86a ± 10.84	0.001

Different letters denote significant differences ($p < 0.05$) between pre-cooking methods

R Raw patties

^a Statistical significance in ANOVA

^b Data expressed as percentage

higher lightness and the lowest chroma among chicken patties, which is consistent with previous findings (Roldán et al. 2014a, b; 2015). Significant differences were also observed for Chroma and Hue, on which a^* and b^* are dependent. The observed changes in color parameters may be caused by a combination of factors, including (i) the cooking loss and ensuing concentration of other meat components, (ii) the denaturation/oxidation of heme pigments and other meat proteins (Estévez and Cava 2004; Ganhão et al. 2010) and (iii) the formation of various colored products generated from the Maillard reaction (Martins et al. 2000). On this line, pre-cooking methods had a significant impact on the formation of MRPs and this influence may actually contribute to explaining the differences already reported for the instrumental color of the samples. In agreement with the yellowness and chroma of pre-cooked samples, GR chicken patties showed the highest amounts of MRPs followed by RT and BL samples. It is known that the formation of Maillard compounds (mainly melanoidins) is affected by the cooking method and cooking temperature (from 55 to 75 °C to initiate reactions) (Ganhão et al. 2010; Ramírez et al. 2005). Considering that the intensity of the Maillard reaction is proportional to the temperature reached during cooking and that contact–heat transfer (as that occurred during grilling) facilitates the formation of MRPs compared to convention heat transfer (occurred during roasting for instance) (Matsuda et al. 2013) the obtained results are reasonable. The formation of these colored compounds after cooking can be a positive factor for acceptance by the consumer (Aaslyng et al. 2007; Berry 1998)

TBARS of chicken patties during pre-cooking, chilling and reheating

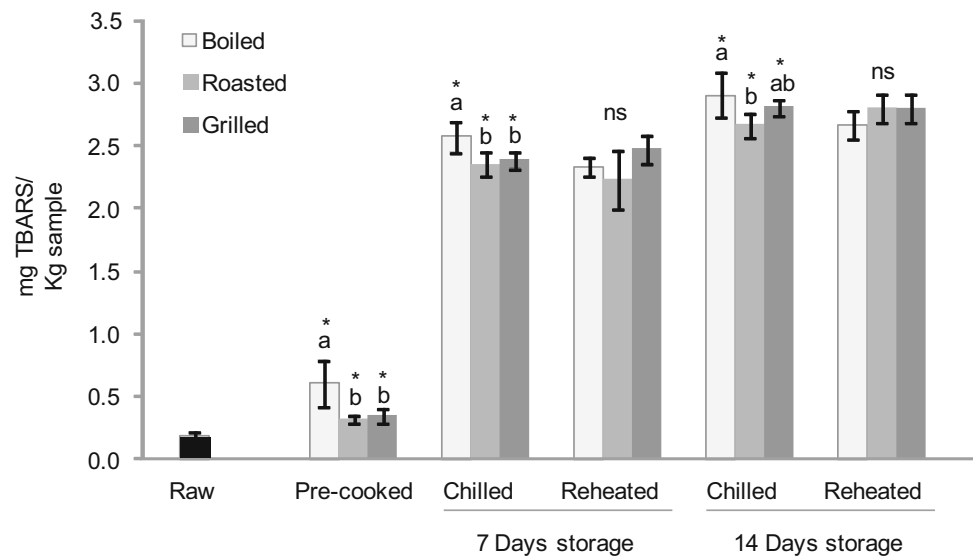
The results for lipid oxidation, analyzed by the formation of TBARS during processing, are showed in Fig. 1. Among pre-cooking methods, BL caused the highest TBARS

numbers (0.6 mg MDA/kg sample), which are consistent with those reported by Weber et al. (2008) in fish fillets (0.8 mg MDA/kg sample) subjected to a similar cooking method. In agreement with the present results are also those reported by Conchillo et al. (2005) in grilled (0.2 mg MDA/kg sample) and roasted chicken breast (0.3 MDA/kg sample).

The highest increase in TBARS values occurred in the first 7 days of chilled storage. TBARS increased 4.25, 7.37 and 6.80-fold times in BL, RT and GR samples respectively. Chilling the samples for 7 additional days increased TBARS only 1.14-fold times on average for all treatments. Again, BL samples had significantly higher TBARS at day 7 than samples subjected to the other pre-cooking methods. Reheating in the microwave at either day 7 or day 14 caused no significant increase of TBARS in any of the samples. Satyanarayan and Honikel (1992) reported that the microwave heating may be the main source of lipid oxidation and impaired quality in fish fillets. In this study, conversely, we observed that the chilled storage of pre-cooked samples had more influence on the increase in TBARS values than the final reheating stage. At the chilling stage, the TBARS values exceeded the limit of consumer acceptability that has been established by several authors between 2 and 3 mg MDA/kg, depending on the meat animal and processing technology applied (Campo et al. 2006; Greene and Cumuze 1982). Above this threshold, rancidity may be perceived by consumers.

The differences between the pre-cooking methods can be explained by the influence of time and temperature in addition to other parameters dependent on the cooking method such as the extent of tissue disruption and the nature of chemical reactions occurred during cooking (Weber et al. 2008). The authors hypothesized whether MDA formed during grilling could be lost by decomposition or by complexation with proteins. While higher TBARS would have been expected in sample subjected to a higher cooking temperature, other factors may have also

Fig. 1 TBARS (mg MDA/kg sample) of raw, pre-cooked, chilled (for whether 7 or 14 days at +4 °C) and reheated chicken patties. Different letters on top of bars denote significant differences ($p < 0.05$) between pre-cooking methods within a particular processing stage. *ns* No significant differences between pre-cooking methods within a particular processing stage ($p > 0.05$). An asterisk on top of a bar denotes significant differences ($p < 0.05$) between that processing stage and the immediately previous one within a particular pre-cooking method



been influential. For instance, BL for 30 min may have caused a higher disruption of cellular compartmentalization in the whole product, facilitating the exposure of membrane lipids to pro-oxidants (Kristensen and Purslow 2001). The physical damage caused in GR samples is presumably limited to the surface while RT samples were subjected to an intense heat treatment for a shorter period of time. The presence of higher amounts of MRPs in RT and GR samples may have also protected lipids against oxidative damage as these products have been found to exert antioxidant properties in meat systems (Serpen et al. 2012). Regardless of the pre-cooking method, all samples had similar TBARS at the final reheating stage.

Color changes in chicken patties during pre-cooking, chilling and reheating

The changes in color parameters (L , a^* and b^*) of chicken patties during processing are shown in Fig. 2. The lightness of BL remained unaffected during cold storage and reheating, while RT and GR samples showed significant modifications ($p < 0.05$), except after reheating upon 7 days of storage (Fig. 2a). The redness decreased in all samples during the subsequent chilled storage (Fig. 2b). After the first 7 days of cold storage, pre-cooking methods showed significant differences ($p < 0.05$) and the GR samples showed the highest redness values. The reheating in microwave did not have any influence on redness, nor at day 7 neither at day 14. The changes in yellowness only affected to some patties at particular processing stages and no common trend was observed (Fig. 2c). The loss of redness and the increase of lightness in chicken patties during chilled storage may be possibly related to the oxidative degradation of denatured globin and the

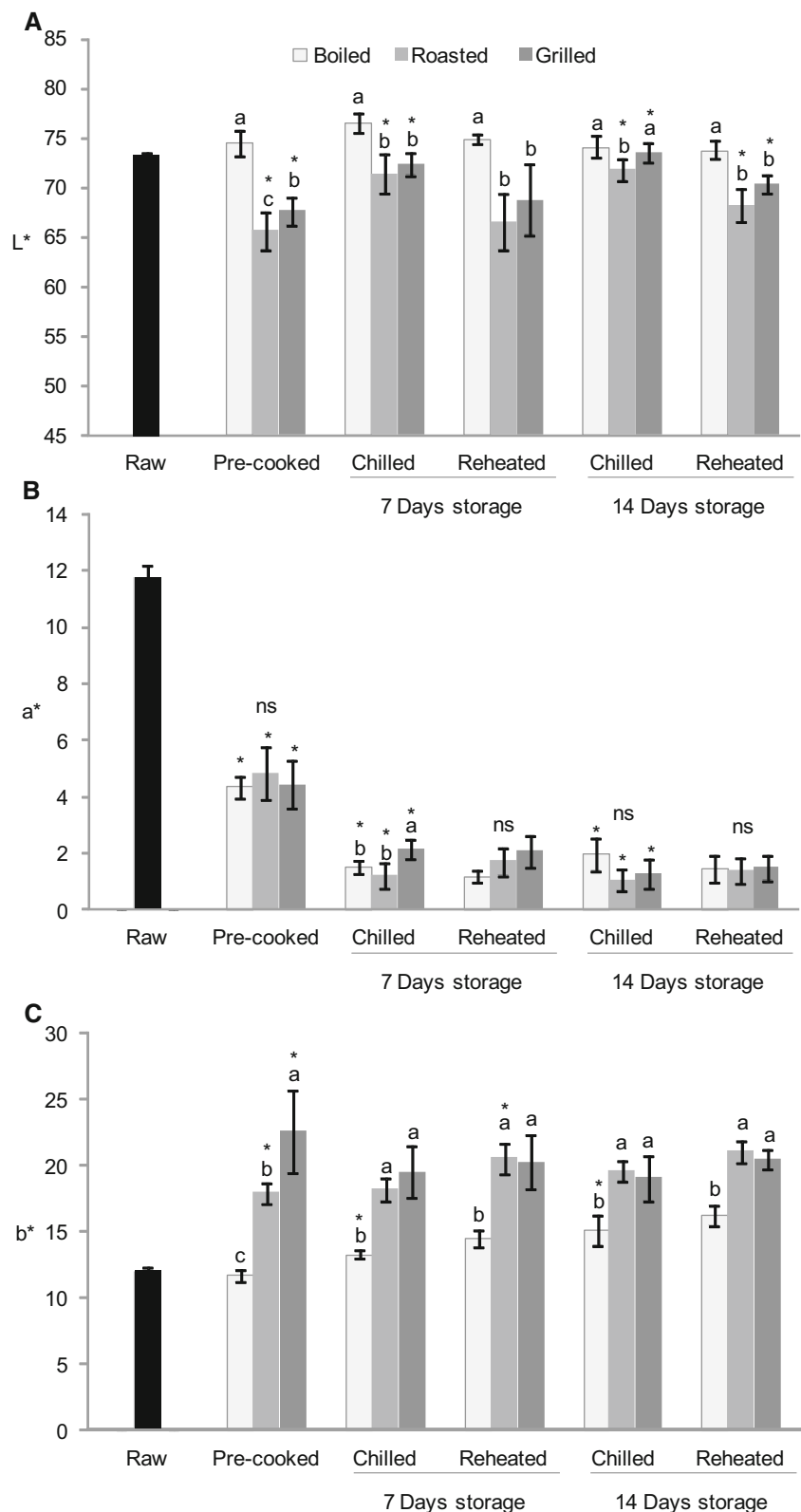
oxidative cleavage of hematin pigment which releases iron from heme molecule (Estévez and Cava 2004). These changes have been already reported and interpreted as an undesirable discoloration of cooked meats subjected to chilled storage (Ganhão et al. 2010).

Odor-active volatiles in chicken patties during pre-cooking, chilling and reheating

Among the total volatiles found in the headspace of the chicken patties during pre-cooking, chilling and reheating, selected lipid-derived carbonyls and Maillard-derived compounds were analyzed (Fig. 3). The carbonyls, namely, hept-(*E*)-enal, Oct-(*E*)-enal, deca-2,4-di-(*EE*)-enal, heptan-2-one, octan-2-one, 3-hydroxy-butan-2-one and octane-2,3-dione (Fig. 3a) have been highlighted by many previous authors as reliable indicators of the oxidative damage to muscle lipids (Estévez et al. 2003; Konopka et al. 1995; Madruga et al. 2009; Roldán et al. 2014a, b). In general, each stage led to a significant increase of this group of volatile compounds in the headspace of the chicken patties, reflecting that lipids suffered successive oxidative degradation during processing. With the exception of reheated samples after 7 days of chilled storage, BL samples had significantly higher amount of lipid-derived volatiles than samples subjected to the other two pre-cooking methods during the subsequent chilling and reheating stages. The more intense lipid oxidation in BL samples reflected in the TBARS values, is now confirmed by the analysis of lipid-derived volatiles.

The increase of particular lipid-derived volatiles during refrigerated storage of pre-cooked meats has been related to the onset of undesirable odors typically described Warmed-Over Flavor (WOF). This hypothesis was in fact

Fig. 2 Evolution of lightness (a), redness (b) and yellowness (c) of pre-cooked, chilled (for whether 7 or 14 days at +4 °C) and reheated chicken patties. Different letters on top of bars denote significant differences ($p < 0.05$) between pre-cooking methods within a particular processing stage. *ns* No significant differences between pre-cooking methods within a particular processing stage ($p > 0.05$). An asterisk on top of a bar denotes significant differences ($p < 0.05$) between that processing stage and the immediately previous one within a particular pre-cooking method



proposed above to explain the higher TBARS values in BL samples than in the patties subjected to the other pre-cooking methods. Interestingly, temperatures above

100 °C, as applied to RT and GR treatments, are indicated as inhibitors of the development of WOF (Bailey and Um 1992). In fact, cooking at temperatures above 100 °C led,

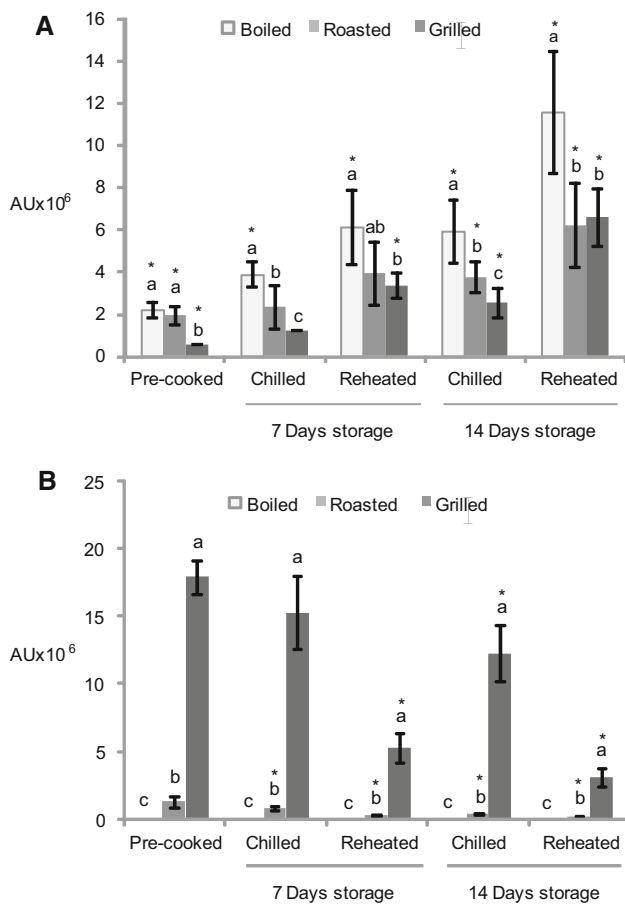


Fig. 3 Odor-active volatiles (Area Units, AU $\times 10^6$): lipid-derived carbonyls (a) and Maillard-derived compounds (b) of pre-cooked, chilled (for whether 7 or 14 days at +4 °C) and reheated chicken patties. Different letters on top of bars denote significant differences ($p < 0.05$) between pre-cooking methods within a particular processing stage. ns No significant differences between pre-cooking methods within a particular processing stage ($p > 0.05$). An asterisk on top of a bar denotes significant differences ($p < 0.05$) between that processing stage and the immediately previous one within a particular pre-cooking method

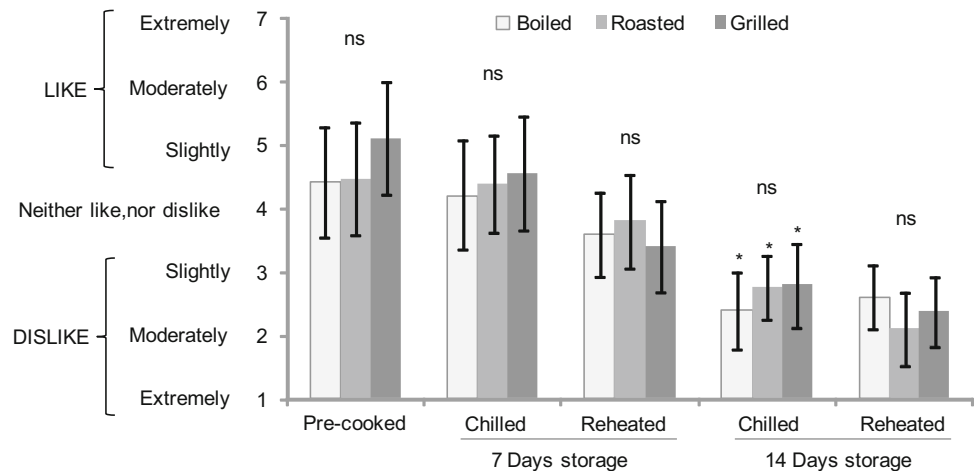
in general, to a lower formation of lipid-derived volatiles and an enhanced formation of other group of volatiles derived from the Maillard reaction. The formation of Strecker aldehydes, namely, methyl propan-2-al, methyl butan-2-al, methyl butan-3-al and benzaldehyde, was more intense in GR samples, followed by far, by RT samples (Fig. 3b). No trace of these compounds was found in the headspace of BL samples. These Strecker aldehydes, formed as a result of the oxidative deamination of free amino acids in the presence of dicarbonyls, are typical volatile components of cooked meats (Domínguez et al. 2014; Estévez et al. 2003; Estévez et al. 2011; Roldán et al. 2014a, b). Methyl butan-2-al and methyl butan-3-al, found in large quantities in GR samples, are potent odorants contributing specific and generally pleasant flavor notes (Campo et al. 2006; Madruga et al. 2009). The highest

concentration of Strecker aldehydes, found in the headspace of freshly pre-cooked GR and RT samples, decreased during the following processing stages with a remarkable loss of volatiles in the final reheating stage. Interestingly, WOF development in pre-cooked meats is not only believed to be the result of an increase of lipid-derived volatiles; a concurrent loss of meaty flavor is also playing a role (Byrne et al. 2002; Campo et al. 2006). Byrne et al. (2001a, b) attributed the reduction in ‘meatiness’ to the loss of sulphur-containing amino acids and other sulphur compounds. This event was confirmed in the present samples through the analysis of free thiols (data not shown). The evolution of Strecker aldehydes, formed during pre-cooking at $T > 100$ °C, and partially lost during the subsequent processing may have also contributed to impair the odor and flavor of patties since this Maillard-derived volatiles have been reported to impart meat aromas (Pegg and Shahidi 2012). These volatile components and their evolution during processing may have an influence in the odor liking and overall acceptance of the products as described below.

Odor liking of chicken patties during pre-cooking, chilling and reheating

The odor liking of the chicken patties was assessed at all stages of processing (Fig. 4). Overall, no significant differences ($p > 0.05$) between the types of pre-cooking (BL, RT and GR) were found at any of the stages under study (pre-cooking, chilling and reheating). Though not statistically significant, the higher scores received by freshly pre-cooked GR samples may be related to the lower extent of lipid oxidation and a more balanced volatile profile in this samples. The scores given by the panelists to the samples successively decreased during processing, reflecting a deterioration of the aroma that may be related to the development of WOF, as supported by the arguments previously reported (“Odor-active volatiles in chicken patties during pre-cooking, chilling and reheating” Section). The decrease of odor liking reached a significant extent after 14 days of refrigerated storage (+4 °C) in all samples irrespective of the pre-cooking method. According to the present results, the scores provided by the panelists shifted over time, from like slightly/neither liked, nor disliked to disliked slightly/moderately disliked. Other authors have also reported an impairment of the aroma profile of cooked meats during subsequent chilled that was generally linked by trained panelists to lipid oxidation, rancidity and WOF (Byrne et al. 2001a, b; Konopka et al. 1995; Mohammad Nisar et al. 2010). However, there is little information on how these changes can actually affect the consumers liking and acceptability. While it is proven that odor liking decreases with storage time and processing

Fig. 4 Odor liking of pre-cooked, chilled (for whether 7 or 14 days at +4 °C) and reheated chicken patties. *ns* No significant differences between pre-cooking methods within a particular processing stage ($p > 0.05$). An asterisk on top of a bar denotes significant differences ($p < 0.05$) between that processing stage and the immediately previous one within a particular pre-cooking method



stages, the relationship between consumers' perception and chemical changes analyzed in the present study is not straightforward. The significant differences observed between pre-cooking methods in terms of TBARS and volatiles were not reflected in the sensory analysis. Also the remarkable differences in MRPs may have had an influence since many of these compounds act as free radical scavengers and have been proposed to control, specifically, the deterioration of flavor in pre-cooked meats (Byrne et al. 2002). Additional chemical analysis or further sensory assessments (i.e. flavor liking) may contribute to a better understanding of these complex phenomena.

Conclusions

The present results showed the relevance of the refrigerated storage and its length, on the chemical deterioration of pre-cooked chicken patties. According to the TBARS numbers and the loss of redness, refrigerated storage has proven to be more influential on lipid oxidation than reheating in a microwave. Selecting a particular pre-cooking method has an impact on the extent of lipid oxidation, the color of patties and the aroma profile of RTE chicken patties. It seems unavoidable to control the storage conditions for inhibiting the development of oxidative reactions in RTE meat products. Further studies will be designed to check the effectiveness of antioxidant strategies (modified atmosphere packaging and phytochemicals) against lipid oxidation and consumer rejection.

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Oxidative degradation of essential amino acids and impaired in vitro protein digestibility in ready-to-eat (RTE) chicken patties

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Running title: “Protein oxidation and nutritional loss in ready-to-eat chicken”

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Abstract

The oxidative damage to proteins during processing of ready-to-eat chicken patties and its impact on the nutritional value and texture properties of the product, were assessed. The effect of pre-cooking techniques (boiling, roasting and grilling), and microwave reheating (600 mW/1min) after 7 and 14 days of cold storage (+4°C) were studied. Cooking methods had an effect ($p < 0.05$) on most protein oxidation parameters, including protein depletion of tryptophan, thiols, protein carbonylation and formation of crosslinks. Overall, the succeeding chilled storage led to an aggravation of the oxidative damage which occurred along with hardness increase and impaired digestibility. Microwave reheating had contradictory effects on protein oxidation parameters. An increase in protein carbonylation occurred along with a cleavage of disulphide bonds that would explain a decrease in hardness. The present study illustrates to which extent the oxidative damage to meat proteins affects protein quality and nutritional value of a severely processed meat product.

Keywords: Convenience food; Protein oxidation; Thiols; Tryptophan; Carbonyls; Cross-linking; Hardness

INTRODUCTION

The consumption of ready-to-eat (RTE) products, especially those derived from poultry meat, has become increasingly popular over the years. This type of convenience food is frequently consumed in fast food restaurants. In USA, for instance, 82.6% of consumers frequent fast food restaurants at least once a week (Statistica, 2014). The high demand for RTE foods may be partially ascribed to the inclusion of women in the labor market and a shift in consumer's appreciation of traditional home-made dishes. Chicken patties are very popular meat products and widely consumed worldwide. However, the processing conditions, that may involve several successive stages such as heating, cold storage and reheating, are responsible for the increasing susceptibility of meat components to suffer oxidative degradation, leading to a remarkable quality deterioration.

Protein oxidation is a chain reaction that leads to the chemical modification of food proteins with overall negative consequences on the food system (Soladoye et al., 2015). The formation of carbonyl compounds, degradation of tryptophan, histidine, methionine and cysteine, loss of sulfhydryl groups and crosslinking formation are some of the most common expressions of the chemical damage to oxidized proteins (Stadtman & Levine, 2003). In processed meat products, the oxidation of proteins results in loss of nutritional quality and protein functionality (Xiong, 2000; Soladoye et al., 2015). Several studies indicate that protein digestibility is compromised as a result of the oxidative degradation of proteins ranging from 20% reduction in oxidized soy protein (Chen et al., 2009) to up to 70% digestibility loss in cooked meat (Santé-Lhoutellier et al., 2008). The loss of protein digestibility hinders the subsequent intestinal absorption of small peptides and amino acids leading to a loss of nutritional value (Estévez, 2011; Soladoye et al., 2015). Besides nutritional aspects, protein

oxidation also affects the texture of meat products (Liu; Xiong & Butterfield, 2000). During the oxidative process, protein cross-links are formed, which increases the hardness of meat and meat products (Estévez, Ventanas, & Cava, 2005; Lund, Lametsch, Hviid, Jensen, & Skibsted, 2007). Additionally, the accumulation of particular protein oxidation products during processing and digestion of muscle foods may also involve certain health risks due to the potential toxicity of these species (Soladoye et al., 2015; Estévez, 2015; Estévez & Luna, *in press*).

Several studies have focused on the effect of cooking methods on lipid oxidation parameters, whereas the influence of cooking techniques on the extent and nature of protein oxidation in meat systems has been scarcely covered. Nevertheless, it is known that processing conditions, storage time and culinary procedures may have a direct effect on lipid and protein oxidation of the meat (ABDEL-KADER, 1996; BYRNE et al., 2002; ESTÉVEZ et al., 2003; NIETO et al., 2013; RAMÍREZ; CAVA, 2005; UTRERA; PARRA; ESTÉVEZ, 2014). Furthermore, microwave reheating is commonly applied to RTE prior consumption and that can further promote chemical alterations in proteins. These chemical changes as well as their consequences for the quality and nutritional value of RTE foods need to be RTE chicken patties and their impact on texture and protein digestibility.

2. MATERIAL AND METHODS

2.1. Chemicals and reagents

All chemicals and enzymes were supplied from Panreac (Panreac Quimica, S.A., Barcelona, Spain), Merck (Merck, Darmstadt, Germany), Sigma Chemicals (Sigma-

Aldrich, Steinheim, Germany) and Scharlau (Gillman, South Australia). Water used was purified by passage through a Milli-Q system (Millipore Corp., Bedford, MA, USA).

2.2. Processing of chicken patties

The processing of the chicken patties was described in a previous work (Ferreira et al., 2016). Burger patties were prepared with 80% chicken thigh muscles, 2% sodium chloride, and 18% of cold water. Round patties (10 cm diameter and 1 cm thickness) were molded and subjected to three different pre-cooking methods: boiling (samples were placed into plastic bags and cooked in a hot water bath to an internal temperature of 80°C for 30 minutes); grilling (using an electric grill to an internal temperature of 250 °C for 4 minutes each side); and roasting (in an oven to an internal temperature of 170°C for 16 minutes). The cooked chicken patties, wrapped with oxygen-permeable PVC films (oxygen permeability: $\sim 17 \text{ cm}^3/\text{m}^2 \text{ day atm}$; moisture permeability: $< 5 \text{ g}/\text{m}^2 \text{ day}$; Tecnodur S.L., Valencia, Spain) were storage under refrigeration (+4 °C) for 14 days. At sampling times (7 and 14 days), samples were reheated in a microwave (600mW/1min; Daewoo, Mod. KOR-6L75; Daewoo Electronics Sales UK Ltd). The whole processing was replicated four times. Pre-cooked patties were analyzed for their proximate composition according to AOAC methods (AOAC, 2000). Samples at each of the processing stages (raw, pre-cooked, chilled and reheated) were subjected to the following additional analyses.

2.3. Methods

2.3.1. Analysis of free thiols and disulphide bonds

Free thiols and disulphide bonds were analyzed following the methodology of Rysman et al. (2014). One g of sample was homogenized with 25 mL of 6 M guanidine

hydrochloride (GuHCl) in 100 mM Tris buffer (pH 8.0) using an Ultra Turrax. The homogenates were centrifuged (for 20 min at 5000 g and 4 °C), and supernatants were filtered (qualitative filter paper, particle retention of 11 µm). The protein concentration of the filtrates was determined spectrophotometrically at 280 nm using a five-point standard curve prepared from bovine serum albumin (BSA). Hence, an aliquot of 3 mL of filtrate was subjected to disulfide reduction by adding 50 µL of 1-octanol and 100 µL of freshly prepared 30% (w/v) sodium borohydride in 1 M NaOH. After incubation at 50 °C for 30 min, an aliquot of 1.35 mL of 6 M HCl was added, followed by stirring for 10 min. Free and total thiols were determined with 4,4'-dithiodipyridine (4-DPS) in the non-reduced and reduced filtrates, respectively. An aliquot of 500 µL of filtrate was mixed with 2 mL of 6 M GuHCl in 1 M citric acid buffer (pH 4.5) and 500 µL of 4-DPS solution (4 mM 4-DPS in 12 mM HCl). The absorbance was measured at 324 nm against 6 M GuHCl in 1 M citric acid buffer (pH 4.5) before the addition of 4-DPS (A_{pre}) and after 30 min of reaction with 4-DPS in the dark at room temperature (A_{post}). A mixture of 2.5 mL of 6 M GuHCl in 1 M citric acid buffer (pH 4.5) and 500 µL of 4-DPS solution was prepared as a blank sample (A_{blank}). The absorbance corresponding to the thiol concentration was calculated by subtracting A_{pre} and A_{blank} from A_{post} . The thiol concentration was calculated on the basis of a five-point standard curve ranging from 2.5 to 500 µM L-cysteine in 6 M GuHCl in 1 M citric acid buffer (pH 4.5). The thiol content was expressed as nmol of thiols per mg of protein. The disulfide content was calculated as half of the difference between total and free thiols.

2.3.2. Tryptophan analysis

The concentration of tryptophan was measured using fluorescence spectroscopy according to the methodology reported by Estévez, Kylli, Puolanne, Kivikari, &

Heinonen, (2008) with some modifications. Samples of chicken patties were homogenized (1:5 w/v) in sodium phosphate buffer pH 6.0 with 8 M urea using an Ultra Turrax. 1.5 mL of 20 mM sodium phosphate buffer pH 6.5 with 6 M GuHCl was added to a 0.5 mL aliquot of the homogenate and stirred in vortex. After dilution (1:1000 v/v) samples were transferred to a 4 mL quartz cuvette with four flat walls (101-QS 10x10mm, Analytics Hellma). The emission spectrum for tryptophan fluorescence was recorded between 300 and 400 nm wavelength with excitation set at 283 nm (Perkin-Elmer LS 55 Luminescence spectrometer, Beaconsfield, UK). The excitation and emission slit widths were set at 10 nm and data were collected at 500 nm per minute. Tryptophan content was calculated on the basis of a five-point standard curve of N-acetyl-L-tryptophan amide (NATA) ranging from 0.1 to 0.5 μ M. Results were expressed as mg NATA equivalents per 100 g of sample.

2.3.3. Analysis of protein carbonyls

Oxidation of proteins was evaluated from the measurement of total carbonyl compounds of the samples after derivatization with 2,4-dinitrophenylhydrazine (DNPH) according to the methodology of Ganhão, Morcuende, & Estévez, (2010) with some modifications. Samples were homogenized with 20 mM phosphate buffer with 0.6 M NaCl pH 6.5 (1:10, w/v). 150 μ L of this homogenate was used to determine the protein concentration and the content of carbonyl compounds. In both cases, the proteins were precipitated with 1mL of 10% trichloroacetic acid (TCA) after centrifugation in the cold (4°C) to 2400 g. For the determination of carbonyl compounds 1 mL of 0.2% DNPH in 2 N HCl was added. For the concentration of protein 1 mL of 2N HCl was added. After incubation at room temperature for 1 hour, proteins were precipitated again with 1mL of 10% TCA and centrifuged at 9000g for 10 minutes. After two washes with 1 mL of

ethanol/ethyl acetate (1:1, v/v) followed by centrifugation at 9000g for 5 minutes, the precipitated proteins were dissolved in 1.5 mL of 20 mM phosphate buffer (pH 6.5) with 6 M GuHCl. Protein concentration of samples was calculated from the absorbance read at 280 nm using a five point standard curve of BSA. The amount of carbonyl was expressed as nmol of carbonyls per mg of protein using a molar extinction coefficient of hydrazones ($21.0 \text{ nM}^{-1} \text{ cm}^{-1}$) with the absorbance read at 370 nm.

2.3.4. Analysis of Schiff bases

The measurement of fluorescence emitted by Schiff base structures was performed with fluorescence spectroscopy according to the methodology reported by Estévez, Kylli, et al., (2008) with some modifications. Sample homogenates were prepared following a similar procedure to that previously described for tryptophan analysis. After dilution (1:20 v/v) samples were transferred to a 4 mL quartz cuvette with four flat walls (101-QS 10x10mm, Analytics Hellma). The emission spectrum for the Schiff base was recorded between 400 nm and 500 nm wavelength with excitation set at 350 nm (Perkin-Elmer LS 55 Luminescence spectrometer, Beaconsfield, UK). The excitation and emission slit widths were set at 10 nm and data were collected at 500 nm per minute. The results were expressed as units of fluorescence intensity emitted by Schiff base structures at 450 nm. These values were corrected according to the protein concentration of each sample by applying a correction factor ($C_f = P_t/P_p$) where P_t is the total average of the amount of protein from all samples and P_p is the content of protein in each type of sample.

2.3.5. Texture measurement

Texture profile analysis was carried out at room temperature with a TA-XT2i Texture Analyzer (Stable Micro Systems, Surrey, UK). Four cylindrical samples of each treatment were taken with a cylindrical cutter (2.5 cm diameter, height of 1.0 cm approximately). The cutter was perpendicularly placed on the patty surface and pressed straight down so that the entire cutter blade was filled up and then lifted up to remove the sample. Samples were subjected to a two-cycle compression test. Analyses were performed in triplicate on each sample. The samples were compressed to 40% of their original height with a cylindrical probe of 5 cm diameter and a cross-head speed of 5 mm/s. The following parameters were determined according to Bourne (1982): hardness (N), adhesiveness ($N \times s$), chewiness (N), springiness (dimensionless), cohesiveness (dimensionless), and resilience (dimensionless). Although all the above-mentioned parameters were determined, only results of hardness are shown.

2.3.6. Analysis of *in vitro* protein digestibility

Protein digestibility was determined *in vitro* by a sequential exposure to digestive proteases following the procedure described by Santé-Lhoutellier et al. (2008) with minor modifications. Samples were homogenized in 100 mM phosphate buffer with 0.6 M NaCl pH 6.5 (1:10, w/v). Proteins were first precipitated by 10% TCA and centrifugation (5000 g, 5 min., +4°C) and then washed in 33 mM glycine buffer at pH 1.8 (gastric pH). Proteins (concentration adjusted to 1 mg/mL) were digested first by pepsin from porcine gastric mucosa (CAS: 9001-75-6; Sigma-Aldrich, Steinheim, Germany) (20 U/mg protein) for 1 h at 37 °C. Digestion was terminated by addition at various times (0, 10, 20, 30, 40, 60 min.) of 15% (final concentration) of TCA and a

subsequent centrifugation at 5000 g for 5 minutes. The content of hydrolyzed peptides/amino acids in the supernatant was estimated by absorbance at 280 nm and the rate of proteolysis was expressed in optical density units by hour ($\Delta OD/\text{hour}$).

The protein pellets obtained upon the centrifugation of the 60 min pepsin hydrolysate were then washed twice in 33 mM glycine buffer at pH 8 (duodenal pH) and the concentration of protein calculated in the same buffer. Proteins were then hydrolyzed by a mixture of trypsin from porcine pancreas (CAS: 9002-07-7) and α -chymotrypsin from bovine pancreas (9004-07-3; Sigma-Aldrich, Steinheim, Germany) for 30 min at 37 °C (6.6 U and 0.33 U/mg protein, respectively). Digestion was terminated by addition at various times (0, 5, 10, 20, 30 min.) of 15% (final concentration) of TCA and the rate of proteolysis was determined as previously described.

2.4. Statistical analysis

Four ready-to-eat chicken patties per treatment and sampling point were prepared in corresponding replicated processes and used as experimental units. The effects of the pre-cooking method and the processing stages (pre-cooking, chilled storage and reheating) on each measured parameter were individually analyzed by corresponding one-way analyses of variance (ANOVA) and Tukey's tests. The experimental unit was computed as co-variable and then discarded for being no influential. Pearson correlations between protein oxidation parameters and quality traits were also calculated using SPSS software (v 19.0). A significance level of $p < 0.05$ was considered.

3. RESULTS

3.1. General composition of pre-cooked chicken patties

Roasted samples presented the lowest moisture value (67.66%), followed by the grilled samples (68.82%) and the boiled counterparts (71.33%). Roasted patties had significantly higher protein content (17.30%) than boiled (16.10%) and grilled patties (16.18%). Regarding to lipid content, roasted (10.87%) and grilled patties (11.71%) had lipid contents than boiled samples (9.08%). These results may be attributed to different losses of water during pre-cooking.

3.2. Loss of free thiols during chicken processing

Results obtained for free thiols are displayed in Figure 1. The pre-cooking led to a significant loss of free thiols and significant differences were found between pre-cooking methods. The roasted and boiled samples showed significantly lower free thiols content than the grilled counterparts. A further loss of thiols was observed during the following chilled storage of pre-cooked patties regardless of the length of such storage. After seven days of cold storage, once again, roasted and boiled patties had lower free thiols than grilled patties. Similar results were found in samples subjected to 14 days of chilled storage. Conversely, the reheating stage did not affect the concentration of thiols in pre-cooked samples. In fact, an increase of free thiol was observed in roasted patties upon reheating after 7 and 14 days of chilled storage. Overall, there was a reduction of approximately 50% in free thiol content between raw (~65 nmol/mg protein) and microwave reheated after 14 days of cold storage (~30 nmol/mg protein).

3.3. Loss of tryptophan during chicken processing

The loss of tryptophan (Figure 2) after the processing of chicken patties (pre-cooked samples) was more pronounced in boiled samples (~40%) than in the roasted and grilled counterparts (10% and 12%, respectively). The following chilled storage, for whether 7 or 14 days, caused a significant decrease of tryptophan in roasted and grilled samples. Reheating in microwave had no effect ($p>0.05$) on the concentration of tryptophan. At chilling and reheating processing stages, the concentration of tryptophan was similar in all samples irrespective of the pre-cooking method applied.

3.4. Formation of protein carbonyls during chicken processing

In general, the formation of carbonyl compounds increased in chicken patties during processing while this effect highly depended on the pre-cooking method applied (Figure 3). Chicken patties subjected to boiling had higher levels of carbonyl compounds compared to raw samples. Grilling and roasting seemed to have no significant effect on the extent of protein carbonylation in chicken patties. Conversely, the following chilled storage for 7 days had a higher impact on grilled and roasted samples than in the boiled counterparts. Prolonging the storage for 14 days significantly promoted the extent of carbonylation in all types of samples. After 7 days of chilled storage and a subsequent reheating, boiled cooked samples had lower values of carbonyl compounds compared to grilled and roasted cooked chicken patties. Similar differences were found between pre-cooking methods for samples stored at 14 days. However, after reheating in microwave, no significant differences ($p>0.05$) were found between cooking methods for the concentration of protein carbonyls.

3.5. Formation of protein cross-linking during chicken processing

The formation of crosslinks during processing of RTE chicken patties was measured by the amount of disulfide bond (Figure 4a) and Schiff base structures (Figure 4b). A remarkable increase in disulphide bond formation was found in pre-cooked samples. This crosslinking was more intense in boiled and roasted chicken patties than in the grilled samples. The effect of storage time on disulphide bond formation was also remarkable in RTE chicken patties. A significant increase of disulphide bond formation was noted after 7 (except for roasted samples) and 14 days of cold storage. At these stages, boiled and roasted samples maintained the highest levels of disulphide bonds followed by grilled chicken patties. With the exception of grilled samples reheated after 7 days of chilled storage, the reheating process led to a significant decrease in the concentration of disulphide bonds in all samples.

The fluorescence emitted by Schiff base structures (Figure 4b) significantly increased from 350 units in raw chicken patties to around 450 units in roasted and grilled chicken samples and to 850 units in the boiled counterparts. After 7 days of cold storage a significant decline in fluorescence was observed in boiled samples while a significant increase was found in the roasted and grilled counterparts. The microwave reheating did not influenced cooked samples stored for 7 days. However, after 14 days of storage, an increment of Schiff base structures was found in boiled and roasted samples. At most processing stages, boiled samples had a higher extent of Schiff base formation than samples subjected to the other pre-cooking methods.

3.6. Texture changes during processing

The modification in the instrumental hardness during the pre-cooking, cold storage and reheating of chicken patties is showed in Figure 5. Among pre-cooked samples, boiled

ones had higher hardness values compared to samples submitted to the other pre-cooking methods. No significant differences were found between roasted and grilled samples after pre-cooking. A significant increase in hardness was found in all samples during the subsequent chilled storage with the exception of boiled samples chilled for 7 days. Reheating process led to a significant decrease of hardness in grilled samples after 7 days of chilled storage and in all pre-cooked samples after 14 days of storage. After 7 and 14 days of cold storage, no differences were observed between the applied cooking methods. It is worth noting that hardness values found after microwave reheating resembled those obtained in pre-cooked samples.

3.7. *In vitro* digestibility changes during processing

The *in vitro* digestibility of proteins from chicken patties during processing was assessed through the analysis of the peptides/amino acids generated upon sequential digestion by pepsin and trypsin/ α -chymotrypsin. Since no clear effects were observed upon application of the pepsin, only results obtained from the *in vitro* digestion by pancreatic enzymes are shown (Figure 6). Pre-cooking significantly increased digestibility of chicken meat proteins compared to raw samples. After pre-cooking, samples submitted to grilling presented the highest digestibility rate while no significant differences were observed between boiled and roasted patties. The subsequent processing stages (chilled storage and reheating) led to successive significant decreases in digestibility with the exception of boiled samples chilled for 7 days. Upon microwave reheating, boiled samples showed the highest values of *in vitro* digestibility followed by the roasted and the grilled counterparts.

4. DISCUSSION

4.1. Impact of protein oxidation on nutritional value of RTE chicken

During oxidative reactions, meat proteins may be attacked directly or indirectly, by reactive oxygen species (ROS) or by-products from lipid oxidation, respectively (Soladoye et al., 2015). These reactions cause changes in the amino acid side chains and the peptide chain, which can lead to protein fragmentation, reticulation, conformational changes, altered susceptibility to proteolytic enzymes, tryptophan losses, increases on carbonyl compounds and formation of protein crosslinking (Chen; Zhao; Sun, 2013; Davies, 2005; Lund et al., 2011; Utrera et al., 2012). Understanding how these severe chemical modifications impact on particular meat quality traits has been one major challenge to food scientists over the last 15 years. Protein oxidation in muscle foods has been linked to altered protein functionality, meat toughening and other impaired sensory attributes (Lund et al., 2011). However, Soladoye et al. (2015) and Estévez (2015) also emphasize the nutritional and health concerns of this phenomenon as the intake of oxidized proteins may lead to an impaired digestion, overall loss of nutritional value and certain health risks. The loss of nutritional value is usually attributed to the depletion of essential amino acids as a result of irreversible oxidative degradations and the resistant of oxidized proteins to be properly hydrolyzed by digestive enzymes (Soladoye et al., 2015). According to the present results, the manufacture of RTE chicken patties involves a considerable loss of protein quality and nutritional value as both phenomena (loss of essential amino acids and impaired digestibility) occurred throughout the processing stages. The oxidation of sulfur-containing amino acids such as methionine during meat processing is of nutritional and health concern. It is worth noting that the loss of thiols in the present samples reached, in some cases, the 70% of the initial concentration in raw samples. On this line, boiled

patties had, at the most advanced processing stage, the lowest concentration of free thiols. Besides the irreversible loss of a minority and essential amino acid, certain methionine oxidation products such as methionine sulfoxide (not quantified in the present study) have been found to exert hepatotoxicity (Dever & Elfarra, 2008). Unlike this thiol oxidation product, the formation of cross-links is reversible under certain circumstances. In fact, microwave reheating led to the cleavage of these bonds that was reflected as an increase in free thiols only in roasted chicken samples. It is understood that thiol oxidation occurred also during reheating by mechanisms different from the formation of disulphide bonds. On the other hand, both tryptophan and their metabolites (kynurenines) play relevant biological functions in target tissues such as brain and the gut (Chen & Guillemin, 2009). Tryptophan is highly sensitive to oxidative degradation in the presence of ROS (Davies, 2005). The loss of tryptophan may be regarded as a relevant nutritional loss since this minority and essential amino acid is remarkably depleted during processing with boiling being the most severe pre-cooking method to this regard (~ 40% loss upon pre-cooking). The loss of tryptophan after cooking process was also observed by Utrera et al., (2014) and Utrera et al., (2012) in beef burgers and Filgueras, Gatellier, Ferreira, Zambiasi, & Santé-Lhoutellier, (2011) in rhea meat. Protein carbonylation is a direct reflection of the oxidative deamination of alkaline amino acids (mainly lysine and leucine) through metal-catalyzed oxidation (MCO) mechanisms (Estévez, 2011). The formation of these carbonyl moieties involves the irreversible loss of the original essential amino acids. Consistently to tryptophan loss, carbonylation was more intense in boiled chicken patties while further processing promoted to a higher extent this reaction in roasted and grilled patties. According to the present study, the cooking method affected the extent of protein oxidation and that may not be fully explained by the combination of time and

temperature but also by the heat transfer mechanisms. While being the less severe pre-cooking method in terms of heat transfer, a prolonged boiling process has been reported to strongly promote lipid oxidation, muscle disruption and the release of free catalytic iron which is required for the MCO of meat proteins (Estévez & Cava, 2004; Estévez, Ventanas & Cava, 2004; Estévez, 2011). The other methods may be more respectful with the muscle structure and temperatures above 100°C promote Maillard reactions that may exert some antioxidant effects against lipid and protein oxidation. In a previous paper we reported significantly higher Maillard products in roasted and grilled patties than in boiled ones (Ferreira et al., submitted). However, these substances may have not contributed antioxidant protection during further processing as particular protein oxidation indexes (i.e. carbonylation) increased remarkably in roasted and grilled samples.

The link between protein oxidation and impaired digestibility has been recurring topic in the food science and nutrition field. After pre-cooking, the digestibility reached a maximum value for all samples. This is generally in agreement with previous findings. Filgueras et al. (2011) reported that the proteolysis rate of meat proteins by pancreatic enzymes increased after cooking. Bax et al. (2012) observed that the oxidation-related protein aggregation occurred during heating above 100 °C improved meat protein overall digestibility in an *in vitro* system. In a further study, Bax et al. (2013) reported that cooking temperature influenced the speed of meat protein digestion, without affecting the efficiency of the small intestinal digestion. According to Sante-Lhoutellier et al. (2007) mild protein oxidation may enhance or not affect protein susceptibility to digestive enzymes, while severe oxidative modifications may reduce protein susceptibility to pancreatic proteases. This could certainly explain the present results as increasing protein oxidation rates at further processing stages (chilling/reheating)

occurred along with a simultaneous decrease in protein digestibility. In agreement with protein oxidation measurements, the protein digestibility of boiled, together with roasted patties was lower than that of grilled patties. At more advanced processing stages, the differences turned to be the opposite, which is also in line with some protein oxidation measurements. Among these, protein carbonylation appeared to be more highly correlated to digestibility values than other protein oxidation measurements ($r=0.76$; $p<0.01$). This may actually be supported by some chemistry fundamentals since trypsin hydrolyses proteins specifically at the carboxyl side of arginine and lysine (Morzel et al. 2006). Likewise, Filgueras et al., (2011) α -chymotrypsin cleaves at locations with methionine and aromatic amino acids. The recognition of the protein cleavage site by trypsin may be negatively influenced by oxidation of basic amino acids and by the presence of carbonyl compounds, whereas the conformational changes of proteins and the oxidation of aromatic amino acids such as tryptophan may affect the activity of α -chymotrypsin. Therefore, it is plausible to consider that meat proteins exposed to high oxidative environments, like those created during application of successive and severe processing stages, display an impaired nutritional value due to loss of essential amino acids and altered digestibility.

4.2. Impact of protein oxidation on texture of RTE chicken

The relationship between protein oxidation and texture changes in meat products has been documented in numerous studies (Estévez & Cava, 2004; Estévez et al., 2005; Ganhão et al., 2010; Lund; Hviid; Skibsted, 2007). Protein crosslinking in meat products is typically identified as responsible for the texture deterioration by promoting the strengthening of the protein structure in the muscle tissue, which leads in turn to meat toughening (Lund et al., 2011). However, the underlying chemical mechanisms

of these rheological alterations have not been fully elucidated. In our experiment, it was noted that protein oxidation was closely related to texture alterations in RTE chicken patties. It is worth mentioning that differences between samples for hardness at the pre-cooking stage may not be explained by moisture as the harder samples (boiled) had significantly higher moisture content than the grilled and the roasted counterparts. On the contrary, the differences between pre-cooked samples for protein carbonyls and Schiff bases were very much in line with the hardness displayed at that stage ($r = 0.809$ and 0.873 , respectively; $p < 0.001$). Schiff bases may be formed as cross-links between carbonyl moieties (including protein carbonyls) and amino groups from alkaline amino acids located in the same or in a different polypeptide chain (Estévez, 2011). Similarly, the increase in hardness of the samples during storage may not be related to storage losses as moisture content did not vary between samples throughout the chilled storage. Again, the oxidation of proteins as analyzed by the concentration of carbonyls and disulphide bonds may have played a role in the increase of hardness but the numerical correlations were, in this case, considerably lower ($r = 0.571$ and $r = 0.484$, respectively; $p < 0.05$). Unexpectedly, reheating led to a decrease in hardness of chicken patties as a probably result of protein conformation changes. The cleavage of the disulphide bonds during reheating, which has no precedent in literature, may be behind the protein changes that may reasonably explain the hardness decrease.

CONCLUSIONS

The present study illustrates to which extent the oxidative damage to meat proteins affects protein quality and nutritional value of a severely processed meat product. Among pre-cooked procedures, boiling seems to be the most damaging on chicken proteins while the succeeding processing stages tend to modify considerably the

differences between pre-cooked samples. Protein carbonylation may not only be a reliable marker of protein oxidation as the oxidative degradation of alkaline amino acids seemed to be linked to the loss of protein digestibility and to some extent, to the modifications in hardness observed during processing of patties. This latter parameter is also driven by the formation and cleavage of protein cross-links. The severe quality changes reported in the present study justifies the application of antioxidant strategies to inhibit protein oxidation in RTE muscle foods. These strategies may be required irrespective of the pre-cooking method and may involve formulation with antioxidant additives and packaging protection during chilled storage.

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FIGURES CAPTIONS

Figure 1. Effect of pre-cooking methods, chilled storage (whether 7 or 14 days at +4°C) and microwave reheating on free thiols in chicken patties.

Different letters on top of bars denote significant differences ($p < 0.05$) between pre-cooking methods within a particular processing stage.

An asterisk on top of a bar at a particular processing stage denotes significant differences ($p < 0.05$) between that processing stage and the immediately previous one within a particular cooking method.

Figure 2. Effect of pre-cooking methods, chilled storage (whether 7 or 14 days at +4°C) and microwave reheating on tryptophan concentration in chicken patties.

Different letters on top of bars denote significant differences ($p < 0.05$) between pre-cooking methods within a particular processing stage. ns: no significant differences between pre-cooking methods at a particular processing stage.

An asterisk on top of a bar at a particular processing stage denotes significant differences ($p < 0.05$) between that processing stage and the immediately previous one within a particular cooking method.

Figure 3. Effect of pre-cooking methods, chilled storage (whether 7 or 14 days at +4°C) and microwave reheating on protein carbonyls in chicken patties.

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Different letters on top of bars denote significant differences ($p < 0.05$) between pre-cooking methods within a particular processing stage. ns: no significant differences between pre-cooking methods at a particular processing stage.

An asterisk on top of a bar at a particular processing stage denotes significant differences ($p < 0.05$) between that processing stage and the immediately previous one within a particular cooking method.

Figure 4. Effect of pre-cooking methods, chilled storage (whether 7 or 14 days at $+4^{\circ}\text{C}$) and microwave reheating on the formation of disulphide bonds (A) and Schiff bases (B) in chicken patties.

Different letters on top of bars denote significant differences ($p < 0.05$) between pre-cooking methods within a particular processing stage.

An asterisk on top of a bar at a particular processing stage denotes significant differences ($p < 0.05$) between that processing stage and the immediately previous one within a particular cooking method.

Figure 5. Effect of pre-cooking methods, chilled storage (whether 7 or 14 days at $+4^{\circ}\text{C}$) and microwave reheating on the hardness of chicken patties.

Different letters on top of bars denote significant differences ($p < 0.05$) between pre-cooking methods within a particular processing stage. ns: no significant differences between pre-cooking methods at a particular processing stage.

An asterisk on top of a bar at a particular processing stage denotes significant differences ($p < 0.05$) between that processing stage and the immediately previous one within a particular cooking method.

Figure 6. Effect of pre-cooking methods, chilled storage (whether 7 or 14 days at $+4^{\circ}\text{C}$) and microwave reheating on the digestibility of chicken patties by trypsin/ α -chymotrypsin.

Different letters on top of bars denote significant differences ($p < 0.05$) between pre-cooking methods within a particular processing stage. ns: no significant differences between pre-cooking methods at a particular processing stage.

An asterisk on top of a bar at a particular processing stage denotes significant differences ($p < 0.05$) between that processing stage and the immediately previous one within a particular cooking method.

Figure 1.

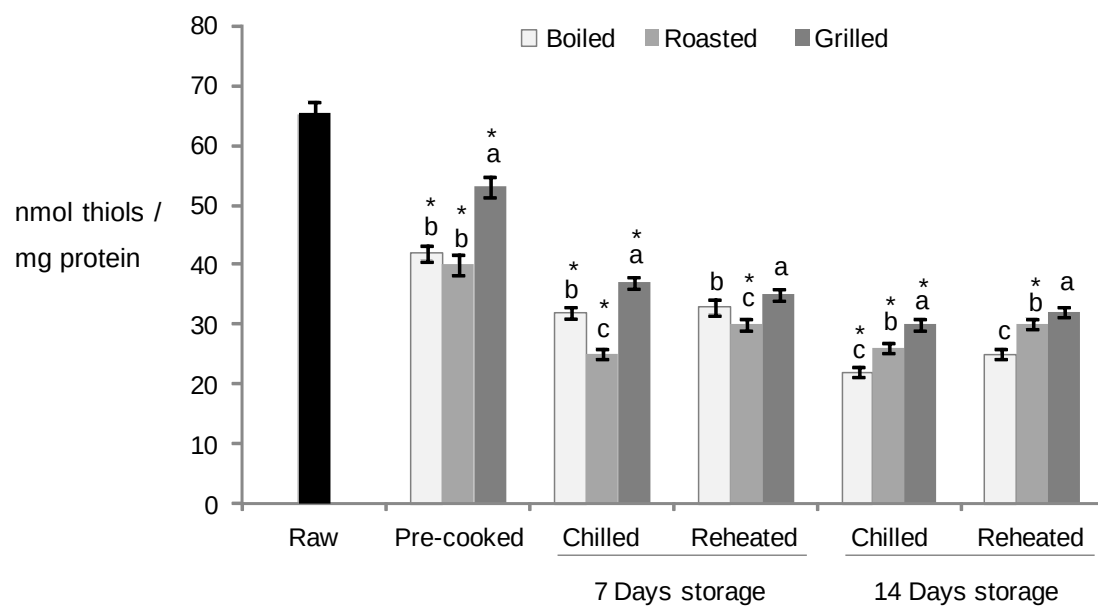


Figure 2.

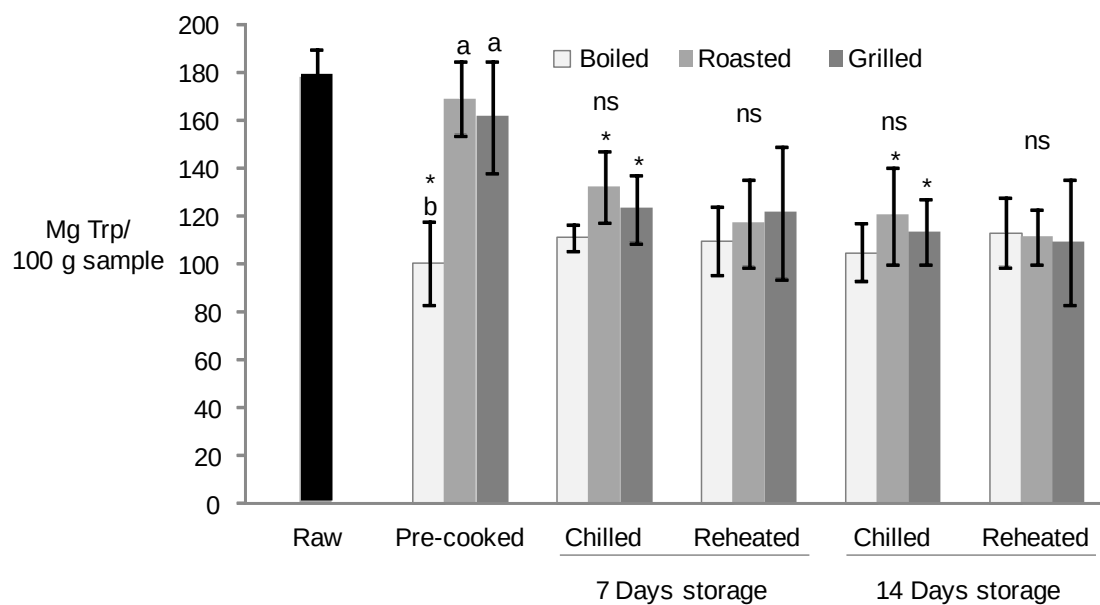


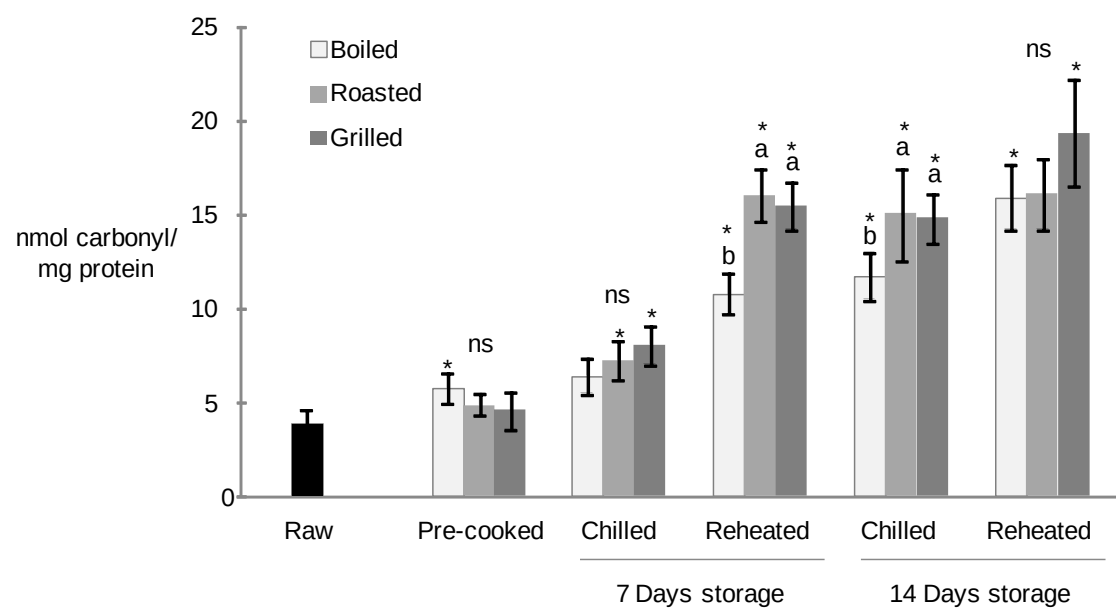
Figure 3.

Figure 4.

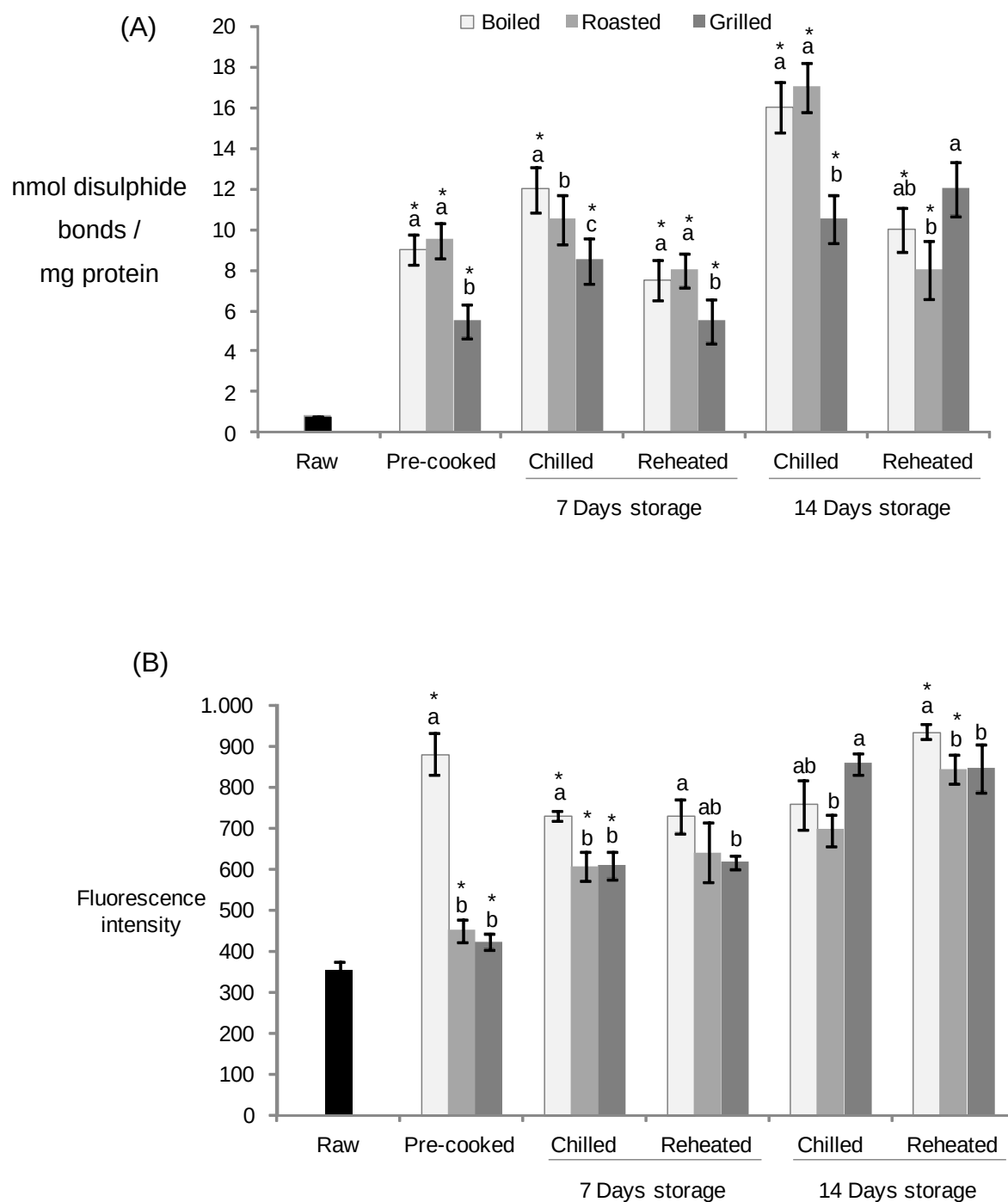


Figure 5.

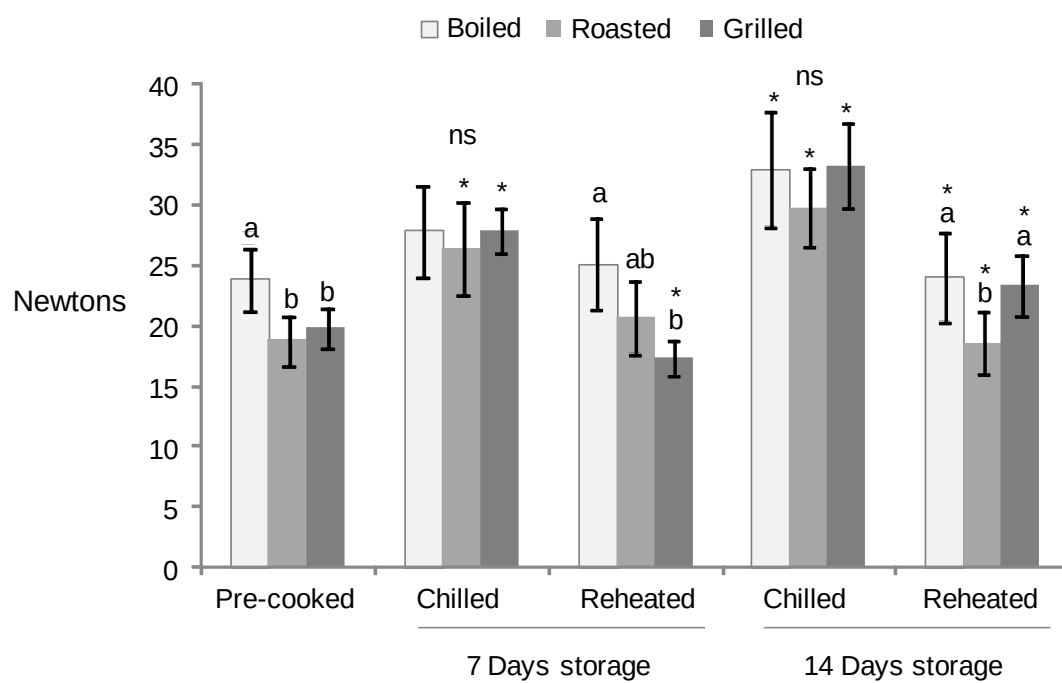
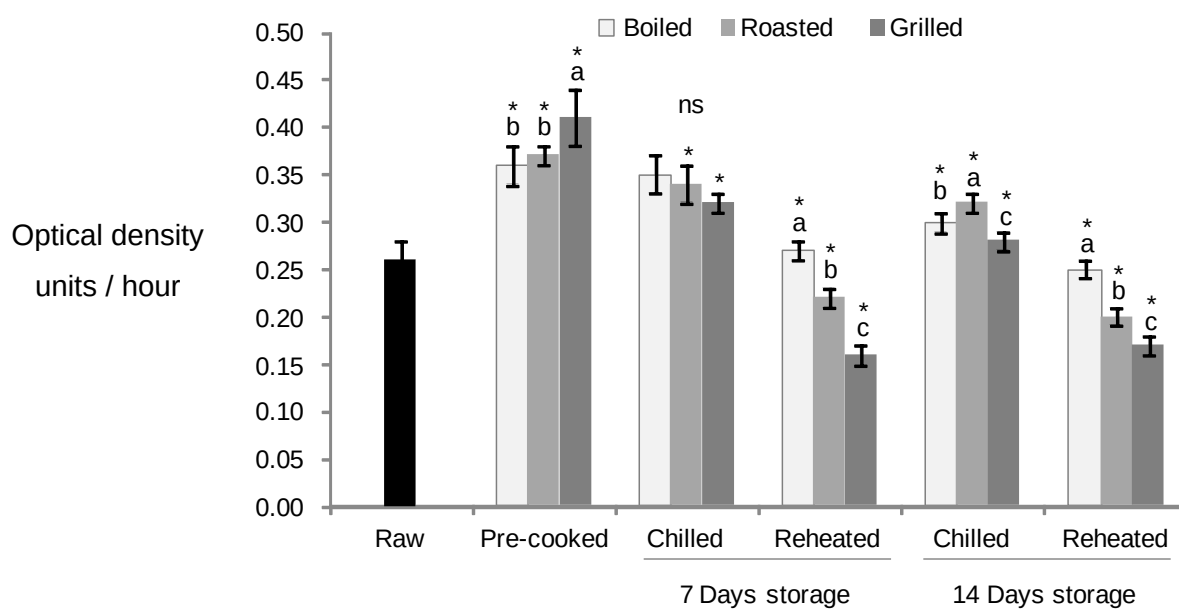


Figure 6.



**Phytochemicals from acorn (*Quercus ilex* L.) inhibit oxidation and
enhance consumer liking of ready-to-eat chicken patties
irrespective of packaging atmosphere**

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Abbreviated running title: "Acorn as antioxidant in convenience food"

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ABSTRACT

This study evaluated the impact of a phenolic-rich acorn extract (200 ppm GAE) and the packaging system (low-oxygen modified atmosphere; 5% vs. normal-oxygen; 21%) on lipid and protein oxidation and consumers liking of the ready-to-eat chicken patties. Samples were subjected to cooking (electric oven, 170°C/16min), cold storage (14 days at 4°C) and reheating (microwave, 600mW/1min). Samples treated with acorn extract kept TBARS numbers and lipid-derived volatiles at basal levels throughout the whole processing irrespective of the packaging type. Consistently, treated patties had lower protein carbonyls than to control ones. The acorn extract also controlled color and texture deterioration during chilled storage and reheating and improved the color and odor liking of the products. Formulating with acorn extract is a feasible strategy to inhibit the oxidation-driven changes and preserve the quality of reheated samples as if there were freshly cooked. The packaging system was irrelevant in terms of quality preservation.

Keywords: convenience foods; phytochemicals; lipid oxidation; protein oxidation; MAP

INTRODUCTION

Changes in the lifestyle of the population have led to an increased demand for low-cost convenience foods that may be consumed without elaborate preparation. In addition, the low availability of time to prepare food by the population associated to the development of microwave equipment promoted the increase in ready-to-eat (RTE) food production (Buckley, Cowan, & McCarthy, 2007). RTE meat products such as burger patties are among the most consumed convenience foods worldwide. During processing, several factors such as grinding, mixing, forming, pre-cooking, packaging, storage and reheating may negatively affect the oxidative stability of muscle tissue and deteriorate the quality traits of the final product in terms of nutritional and/or sensory properties (Estevez, 2015; Ganhão, Morcuende, & Estévez, 2010a).

Lipid oxidation promotes the generation of secondary compounds such as pentanal, hexanal and alkadienes responsible for the formation of off-flavors in meat products (Estévez, Morcuende, Ventanas, & Cava, 2003; Jayasena, Ahn, Nam, & Jo, 2013). These volatile compounds are frequently related to rancid aroma formation and are easily detected by consumers because of its low odor threshold (Pegg & Shahidi, 2012). Meat proteins are also susceptible to oxidative deterioration. Protein oxidation leads to carbonylation and crosslinking formation in proteins, which are associated to sensory deterioration of meat by increased hardness and decreased water-holding capacity (Falowo, Fayemi, & Muchenje, 2014; Lund, Lametsch, Hviid, Jensen, & Skibsted, 2007). Oxidized proteins have impaired nutritional value by the loss of essential amino acids and decreased protein digestibility and are thought to display toxic effects on humans (Soladoye et al., 2015).

Meat industry has used assorted strategies in order to minimize the quality degradation of meat products as a result of oxidative reactions. The application of antioxidants and

the use of modified atmosphere packaging (MAP) have been recognized as major strategies against oxidative deterioration of meat and meat products. On the other hand, the toxicological concerns on synthetic antioxidants has led to an increasing interest in natural products with antioxidant properties (Shahidi, 2000). Extracts from numerous herbs, spices, oleaginous, vegetables and fruits have been found to be natural sources of phenolic compounds with antioxidant properties that may be used to preserve food quality (Falowo et al., 2014). The incorporation of natural antioxidants to meat products can be accomplished by means of feed manipulation or food formulation (Estévez, 2011; Falowo et al., 2014). Acorns are commonly used for pig feeding and found to increase efficiently the oxidative stability of pork through the enrichment of muscle tissue in tocopherols (Ventanas et al., 2007). However, this fruit contains a large variety of other phytochemicals with proven antioxidant properties such as ellagitannins that may not be bioavailable and hence, do not protect meat against oxidation (Cantos et al., 2003; Espín et al., 2007). The direct addition of crude extracts from acorns to meat products may be a feasible and costless strategy to protect meat products against oxidation.

Regarding to packaging, the use of high-oxygen MAP is commonly used in fresh red meat cuts to increase shelf-life and improve meat color stability. However, high concentrations of oxygen increases protein and lipid oxidation in meat products (Lund et al., 2007). Considering results obtained in previous studies (Ferreira et al., 2016), removal of oxygen in MAP may be more appropriate for RTE meat products as oxygen provides nothing but oxidative instability. Thus, the aim of this study was to evaluate the effect of the combination of an acorn extract with low-oxygen MAP on the lipid and protein oxidative stability of RTE chicken patties during pre-cooking, cold storage and reheating.

2. MATERIAL AND METHODS

2.1. Chemicals and reagents

All chemical were supplied from Panreac (Panreac Quimica, S.A., Barcelona, Spain), Merck (Merck, Darmstadt, Germany), Sigma Chemicals (Sigma-Aldrich, Steinheim, Germany) and Scharlau (Gillman, South Australia). Water used was purified by passage through a Milli-Q system (Millipore Corp., Bedford, MA, USA).

2.2. Characterization of acorns

Samples of acorns (*Quercus ilex* L. subsp. *Ballota* (Desf.) Samp.) were collected at the stage of full ripeness in the Caceres region, Spain (altitude=450 m) during the winter of 2015. After hand-harvest, the samples were immediately transferred to the laboratory, cleaned and sorted to eliminate damaged and shriveled fruits, and then processed for characterization and extraction of bioactive compounds. The proximate composition (moisture, lipid, crude protein, ashes) of the selected fruits was analyzed according to AOAC methods (AOAC 2000).

2.3. Extraction and characterization of bioactive compounds

For the preparation of acorn extracts, fruits were peeled, finely ground (<1 mm particle size) and lyophilized. Hundred grams of lyophilized acorn was homogenized for 3 min with 1.5 volumes (w/v) of 60% food grade acetone using an Ultra Turrax at 7500 rpm. The homogenates were centrifuged at 600 g for 3 min at 4°C using an Eppendorf centrifuge 5810 R (Eppendorf, Hamburg, Germany). The supernatants were collected and filtered through nº 54 Whatman paper while the residue was re-extracted once

more following the procedure previously described. The two supernatants were combined and subsequently dried using rotary evaporator at 500 mb and $T^a < 30^{\circ}\text{C}$. The extraction procedure (extracting solvent, ratios, conditions etc.) was optimized in a preliminary study to maximize the concentration of bioactive compounds. Residues were brought to 200 mL volume with distilled water and immediately transferred to the laboratory for quantification of total phenolic content (TPC), condensed tannins and radical scavenging activity. TPC was determined following to the Folin-Ciocalteu method described by Singleton and Rossi (1965). Condensed tannins were determined by the butanol-HCl procedure described by Porter et al. (1986). The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay reported by Kähkönen and Heinonen (2003) was employed for the measurement of the radical scavenging activity.

2.3. Experimental setting

Chicken patties were elaborated with chicken thigh and drumstick meat (1:1 w/w) obtained from a local marketplace in the city of Caceres (Spain). Firstly, two groups of samples were considered depending on the addition of acorn extract. The CONTROL group of samples was made using the general basic formulation: 80% meat, 2% sodium chloride and 18% of distilled water. The TREATED group of samples was manufactured using the same formulation with 18% of distilled water being replaced by an equivalent volume of acorn extract to result in a TPC of 200 mg gallic acid equivalents (GAE) per kg of chicken patty. The chicken meat and ingredients were homogenized in cutter (Mainca®, USA) for 6 minutes. Then, chicken patties (~100g) were molded in a burgermaker (10 cm diameter and 1 cm thickness).

After production, chicken patties were subjected to 4 different manufacturing processes as follows: i) raw patties: right after manufacture, raw patties were

transferred to the laboratory for analyses; ii) cooked patties: patties were cooked in an oven (Unox®, Mod. GN2.1, Cadoneghe, Italy) to an internal temperature of 170°C for 16 minutes (8 min each side), allowed to cool down and immediately were transferred to the laboratory for analyses; iii) cooked and chilled patties: after cooking, patties were individually placed in polystyrene trays, wrapped with PVC films (oxygen permeability: $\sim 17 \text{ cm}^3/\text{m}^2 \text{ day atm}$; moisture permeability: $< 5 \text{ g}/\text{m}^2 \text{ day}$; Tecnodur S.L., Valencia, Spain) and stored under refrigeration ($+4 \text{ }^\circ\text{C}$) for 14 days and finally transferred to laboratory for analyses; iv) cooked, chilled and reheated patties: At the end of the chilled storage, cooked and chilled samples were eventually reheated in a microwave (600mW/1min; Daewoo, Mod. KOR-6L75; Daewoo Electronics Sales UK Ltd) and subsequently transferred to laboratory for analyses. Depending on the packaging used during chilled storage, two additional group of samples were considered: NORMAL-OX, packed in normal atmosphere (21% O_2 , 0.5% CO_2 and 78.5% N_2) and LOW-OX, packed in a low-oxygen modified atmosphere (5% O_2 , 27% CO_2 and 68% N_2). The whole experimental setting described above was replicated in independent batches totaling 4 samples per experimental group (**Table 1**).

2.4. Chemical composition and fatty acid profile of patties

Moisture and total protein were determined using AOAC methods (AOAC, 2000). Lipid content of chicken patties was determined using Folch, Lees & Stanley (1959) method. Fatty acid methyl esters (FAMES) were prepared by methylation with a cold methanolic solution of 2 N potassium hydroxide according to Cert, Moreda, & Pérez-Camino (2000) procedures. FAMES were separated using a Hewlett–Packard HP-5890A gas chromatograph equipped with an on-column injector, a flame ionization detector and a capillary column (Supelco®, 50m x 0.32mm internal diameter; 1.0 μm film thickness).

Detector gas flow rate for hydrogen, helium and synthetic air were 35 mL min⁻¹, 15 mL min⁻¹ and 400 mL min⁻¹, respectively. Helium was used as the carrier gas (flow rate 1.7 mL min⁻¹). The initial column temperature was programmed to 180 °C for 5 min, which was then increased to 210 °C at 1 °C min⁻¹. Subsequently, the temperature was increased to 250 °C at 4 °C min⁻¹ and held at the maximum temperature for 10 minutes, resulting in a total run time of 55 minutes. The Supelco® 37 Component FAME Mix (Sigma-Aldrich, USA) was used as a reference standard to identify the fatty acid peaks.

2.5. Thiobarbituric acid-reactive substances (TBARS)

Thiobarbituric acid-reactive substances were extracted in acidic conditions and quantified spectrophotometrically following the method proposed by Ganhão, Estévez & Morcuende (2011). Results were expressed as mg of TBARs per kg of chicken patties.

2.6. Analysis of protein carbonyls

Oxidation of proteins was evaluated from the measurement of total carbonyl compounds of the samples after derivatization with 2,4-dinitrophenylhydrazine (DNPH) according to the methodology of Ganhão, Morcuende, & Estévez, (2010a) with some modifications. Samples were homogenized with 20 mM phosphate buffer with 0.6 M NaCl pH 6.5 (1:10, w/v). 150 µL of this homogenate was used to determine the protein concentration and the content of carbonyl compounds. In both cases, the proteins were precipitated with 1 mL of 10% trichloroacetic acid (TCA) after centrifugation in the cold (4 °C) to 2400 g. For the determination of carbonyl compounds 1 mL of 0.2% DNPH in 2 N HCl was added. For the concentration of protein 1 mL of 2N HCl was added. After incubation at room temperature for 1 hour, proteins were precipitated again with 1mL

of 10% TCA and centrifuged at 9000 g for 10 minutes. After two washes with 1 mL of ethanol/ethyl acetate (1:1, v/v) followed by centrifugation at 9000 g for 5 minutes, the precipitated proteins were dissolved in 1.5 mL of 20 mM phosphate buffer (pH 6.5) with 6 M GuHCl. Protein concentration of samples was calculated from the absorbance read at 280 nm using a five point standard curve of BSA. The amount of carbonyl was expressed as nmol of carbonyls per mg of protein using a molar extinction coefficient of hydrazones ($21.0 \text{ nM}^{-1} \text{ cm}^{-1}$) with the absorbance read at 370 nm.

2.3.4. Objective color measurement

Color measurements were performed by measuring lightness (L^*), redness (a^*) and yellowness (b^*) values in triplicate at different random points on the surface of the chicken patties using a portable Minolta Chromameter CR-300 (Minolta Co., Ltd, Osaka, Japan) equipped with a measuring head (CR-300). Color measurements were made on patties prior to frozen storage at room temperature with an illuminant D65 and a 0° angle observer. The equipment was previously calibrated with the white tile provided by the supplier.

2.3.5. Texture measurement

Texture profile analysis was carried out at room temperature with a TA-XT2i (Stable Micro Systems, Surrey, UK). Four cylindrical samples of each treatment were taken with a cylindrical cutter (2.5 cm diameter and height of 1.0 cm, approximately). The cutter was perpendicularly placed on the patty surface and pressed straight down so that the entire cutter blade was filled up then lifted up to remove the sample. Samples were subjected to a two-cycle compression test. Analyses were performed in triplicate on each sample. The samples were compressed to 40 % of their original height with a

cylindrical probe of 5 cm diameter and a cross-head speed of 5 mm s⁻¹. Hardness (N) was determined according to Bourne (1982) and calculated as a maximum force applied on the first compression peak.

2.3.6. Analysis of volatiles

Volatiles were analyzed in reheated samples by headspace/solid-phase microextraction (HS/SPME) and gas chromatography/mass spectrometry (GC/MS) following the method described by Estévez, Morcuende, Ventanas, & Cava (2003). A gas chromatograph Hewlett–Packard 5890 series II coupled to a mass selective detector Hewlett–Packard HHP-5791A was used. One gram of minced sample was weighed into a 4 mL vial, which were closed with a teflon/silicone septum (National Scientific) and preconditioned for 10 min at 37 °C. An SPME fiber (50/30 µm divinylbenzene-carboxen-polydimethylsiloxane coating) was preconditioned prior analysis at 220 °C during 45 min and then inserted through the septum and exposed to the headspace of the vial. After extraction, the SPME fiber was immediately transferred to the injector of the chromatograph (splitless mode). The separation of volatile compounds was performed on a 5% phenyl–methyl silicone (HHP-5)-bonded-phase fused-silica capillary column (Hewlett–Packard, 50 m × 0.32 mm i.d, film thickness 1.05 µm). The carrier gas was helium at 18.5 psi (flow rate of 1.6 mL min⁻¹) at 40 °C. The SPME fiber was desorbed and maintained in the injection port at 220 °C during the whole chromatography run. The temperature program was isothermal for 10 min at 40 °C and then increased at the rate of 7 °C min⁻¹ to 250 °C and held for 5 min. The GC-MS transfer line temperature was 270 °C. The mass spectrometer operated in the electron impact mode with an electron energy of 70 eV and a multiplier voltage of 1650 V and collected data at a rate of 1 scan s⁻¹ over a range of m/z 40-300. Selected

lipid-derived volatiles were positively identified by comparing its linear retention indexes/mass spectra (LRI/MS) with that from standard compound (Sigma-Aldrich, Steinheim, Germany). Results are provided in arbitrary area units (AU x 10⁶).

2.3.7. Color and odor liking assessment

In order to assess the impact of the acorn extract on sensory properties of the patties, control and treated chicken patties packaged in NORMAL-OX were used for color and odor liking assessment. Color and odor liking analysis of ready-to-eat chicken patties was performed by 50 people (staff and students from Veterinary Faculty in Caceres, Spain, ranging from 23 to 60 in age and regular consumers of chicken products). A hedonic scale of seven points from extremely disliked (1) to extremely liked (7) was used. For color liking was performed by panelists in cylinders (2.5cm diameters and 1.0cm height) to visual evaluation. For odor liking, five grams of each sample were finely minced, dispensed in falcon tubes, sealed and wrapped with aluminum foil and offered to the panelists after being warmed up to 37°C for 10 min in oven. Each sample was coded with three random numbers.

2.4. Statistical analysis

Four RTE chicken patties per treatment and processing stage were prepared in corresponding replicated processes and used as experimental units. The effect of the acorn extract application and packaging on each measured parameter was analyzed by a two-way ANOVA and Tukey's test. A Kruskal-Wallis analysis was applied to sensory data. A significance level of $p < 0.05$ was considered.

3. RESULTS AND DISCUSSION

3.1 Characterization of acorns

The chemical composition and hence, bioactive effects of acorn pulp is highly variable depending on a number of factors such as the species, season, ripeness state and the harvesting location (Rodríguez-Estévez et al., 2008). The results obtained for the present acorns are comparable to results from other authors as the amounts of moisture (36.96 ± 0.16), protein (6.37 ± 0.04) and fat (5.35 ± 0.06) are within a normal range (Daza et al., 2005, Daza et al., 2007). Of particular relevance is the concentration of total phenolic compounds (TPC) as these phytochemicals have been found to display assorted bioactivities including antioxidant potential (Cantos et al., 2003; Tejerina et al., 2012). Our results (928 ± 35 mg GAE/100 g fruit) show an improvement of the extraction yield found by Custódio et al., (2015) and Tejerina et al., (2012) that can be attributed to the extracting solvent. It is worth noting that the procedure used was previously optimized for assorted variables such as polarity of the extraction solvent, extraction time and ratio solvent: sample, among others, as they have been recognized as highly influential in the efficacy of phenolics extraction from fruits (Nacz & Shahidi, 2006). Condensed tannins (61 ± 10 mg equivalent procyanidins B1/100 g fruit) were also quantified in acorns owing to their recognized bioactivity and ability to interact with proteins (Cantos et al., 2003; Espín et al., 2007). The TPC and antioxidant activity displayed by the acorns phytochemicals against the DPPH radical (51.87 ± 3.44 microM Trolox/g fruit) is comparable to that found in berries such as strawberry tree (*Arbutus unedo* L.), azarole (*Crataegus azarolus* L.), blackthorn (*Prunus spinosa* L.) and thornless blackberry (*Rubus ulmifolius* Schott.) (Ganhão, Morcuende, & Estévez, 2010a). Some of these fruits have shown effective protection against lipid and protein oxidation in assorted processed muscle foods (Ganhão, Morcuende, &

Estévez, 2010a, 2010b; Ganhão, Estévez, Armenteros, & Morcuende, 2013; Armenteros et al., 2013) so the antioxidant potential of the present acorns is promising.

3.2 Impact of acorn extract on chemical composition and objective color of RTE chicken patties

The chemical composition (moisture, protein, fat and fatty acid profile) and instrumental color of experimental patties is shown in **Table 2**. No significant differences ($p>0.05$) were observed between CONTROL and TREATED raw samples for moisture, protein and lipid content. As expected, cooked samples had lower content of moisture and higher percentage of protein and lipids compared to raw ones. No differences ($p>0.05$) were found between samples for saturated ($\sum$ SFA), monounsaturated ($\sum$ MUFA) and polyunsaturated ($\sum$ PUFA) fatty acids.

Color parameters (L^* , a^* and b^*) of chicken patties were affected by cooking and the addition of acorn extract. TREATED raw samples showed the highest levels of redness (a^*) and lowest values of yellowness (b^*). Lightness (L^*) of raw samples was not affected by the addition of acorn extract. However, after cooking, a marked decline on L^* value was noted in TREATED samples. Cooked samples showed lower a^* and higher b^* values compared to raw chicken patties. Chroma and hue angle were higher in cooked samples and the addition of acorn extract promoted a decrease in hue angle values. The influence of crude extracts from plants and fruits on the color of muscle foods is a challenge as the consumers may reject a product with an unexpected appearance. Assorted examples of this situation are found in the literature (Ganhão et al., 2010b; Rodríguez-Carpena, Morcuende, & Estévez, 2011). The consumer attitude on the modifications of the objective color of the patties by the extracts is analyzed in due course.

3.2 Lipid and protein oxidation during processing of RTE chicken patties

As a result of the enduring oxidative reactions during processing, CONTROL samples had succeeding increases in TBARS from 0.240 in raw samples to 3.186 mg TBARS kg⁻¹ sample in reheated patties (**Figure 1A**). While high temperatures during cooking promoted lipid oxidation, the most remarkable increase in TBARS was found during the chilled storage of cooked patties. Reheating did not lead to a significant increase in TBARS. Reducing the oxygen level through MAP packaging significantly reduced the extent of lipid oxidation in CONTROL patties while the final reheating tended to equalize the TBARS levels in CONTROL samples regardless of the packaging applied during the previous storage. The effect of the addition of acorn extract on lipid oxidation was particularly remarkable as no significant increase of TBARS was found during the entire process. In TREATED patties, the TBARS values in raw samples were equivalent to those quantified in the samples after cooking, chilling and reheating. Obviously, it worth noting that RTE chicken patties formulated with acorn extract had lower TBARS values than the CONTROL counterparts at all stages (except raw) irrespective of packaging conditions. In fact, the packaging seemed to be irrelevant for controlling lipid oxidation as long as the acorn extract was present.

The formation of protein carbonyls as an expression of the oxidative damage to chicken proteins differed from that observed for TBARS (**Figure 1B**). The processing stages that involved an increase in temperatures, namely cooking and microwave reheating, significantly promoted protein carbonylation in CONTROL chicken patties while no significant increase of protein carbonyls was observed during chilled storage of cooked samples. Significant differences ($p < 0.001$) were observed in carbonyl compounds between TREATED and CONTROL chicken patties at all stages of processing.

Carbonyl content in CONTROL samples (6.73 nmol carbonyl/mg protein) was about two-fold higher than that in the TREATED counterparts (3.79 nmol carbonyl mg⁻¹ protein). CONTROL samples had the highest level of protein carbonyls after microwave reheating irrespective of packaging type. In fact, the type of packaging did not affect the rate of carbonyl formation, which remained between 3.97 and 4.23 nmol carbonyl/mg protein.

Initially, it was postulated that lipid and protein oxidation reactions were initiated by the same factors and hence the strategies used to control lipid oxidation were also used to regulate the oxidative degradation of proteins (Lund, Heinonen, Baron, & Estévez, 2011). The present results, however, suggest that both reactions are governed by different mechanisms as previously reported by Estévez (2011). According to our initial hypothesis, packaging with a low oxygen atmosphere may be a feasible way for controlling lipid oxidation during chilled storage of RTE chicken patties. While this was confirmed to a certain extent, overall, the effect of LOW-OX packaging was eclipsed by the addition of the acorn extract. Protein oxidation was completely unaffected by the packaging strategy which was, to some point, expected as the formation of protein carbonyls may occur in the absence of molecular oxygen (Estévez, 2011). As generally known, oxygen is required for the autooxidation of unsaturated fatty acids but plays, as such, a minor role in the oxidative deamination of alkaline amino acids, that eventually leads to the formation of carbonyl residues in proteins. The present study confirms that reducing oxygen in cooked chicken has no impact on the carbonylation of proteins. On the other hand, high-oxygen MAP has been found to promote protein oxidation in red meat during chilled aging (Lund, Hviid, & Skibsted, 2007). While the meat system and the conditions are fairly different between experiments, it is also likely

that the pro-oxidant effect on proteins described by Lund et al. (2007) would be exerted by oxidizing lipids, highly susceptible in high-oxygen systems.

Many scientific reports have illustrated on the use of phenolic-rich plant and fruit extracts as inhibitors of lipid oxidation and promoters of shelf life and quality in chicken meat products (Estevez, 2015; Bekhit, Hopkins, Fahri & Ponnampalam, 2013, Hwang et al., 2013, Estévez, 2011). Acorns are known for their indisputable benefits as feeds for pigs and broilers in terms of oxidative stability and quality of the derived meat products (Ventanas, Ventanas, Tovar, Garcia, & Estévez, 2007; Boudieroua, et al., 2009). In our experiment, the direct addition of acorn extract was highly effective in the control of lipid and protein oxidative degradation in RTE chicken patties. Furthermore, the addition of acorn extract had more impact on the protection against lipid and protein oxidation than modified atmosphere packaging. The protective effect of acorn extract against oxidative degradation of lipids is probably related with the intense antioxidant activity of polyphenols from acorn. These phytochemical could have also protected proteins against carbonylation. Condensed tannins and other phenolic compounds naturally present in the acorn are known to displaying radical scavenging and metal-chelation activities (Espín et al., 2007). Both modes of action may be applicable to the present results as the formation of protein carbonyls is a metal-catalyzed radical-mediated mechanism (Estévez, 2011). The TBARS levels found in CONTROL samples chilled for 14 days and submitted to microwave reheating reached similar values than those found by other studies (Campo et al., 2006; Greene & Cumuze, 1982). These results have been described as the limit of sensory acceptability by consumers (2.0 to 3.0 mg de TBARS/kg sample) (Pegg & Shahidi, 2012). The consequences of protein oxidation in meat and poultry involve loss of protein functionality, digestibility, increased of meat toughness and the formation of

compounds with potential toxicity for humans (Soladoye, Juarez, Aalhus, Shand, & Estévez, 2015, Estévez & Luna, 2016). Hence, by keeping TBARS at basal levels and efficiently controlling protein oxidation throughout the entire process, acorns extract may deliver some benefits such as the preservation of acceptable sensory, nutritional and health properties of the RTE chicken product.

3.3 Evolution of redness and hardness during processing of RTE chicken patties

The CONTROL samples suffered color changes during processing that are displayed here as modifications in the redness of the meat products (**Figure 2A**). The drop in redness in cooked samples may be ascribed to the brownish pigments, mainly, denatured-globin hemochromes, formed as a result of high temperatures (Fox, 1966). During cooking, the heme protein is denatured and the iron is oxidized into its ferric form whereas the hematine pigment remains intact (Ganhão et al., 2010a). The formation of colored Maillard products upon heating and the physico-chemical state of protein are also influential (Fox, 1966; Ganhão et al., 2010a). The intense red-brownish color of freshly cooked meat is preferred by meat consumers (Berry, 1998) and hence, color changes occurred during further processing may affect consumer's acceptability. CONTROL samples certainly suffered a significant loss of redness during chilled storage that was significantly diminished by the LOW-OX packaging. The oxidative degradation of pigments in cooked meats has been proposed as main responsible for this quality deterioration (Ganhão et al., 2010a). The present results support this hypothesis because the addition of a phenolic-rich extract of acorn with proven antioxidant activity was able to protect the color of freshly made chicken patties that remained stable until the final reheating stage. Once again, the effect of the packaging strategy was negligible in samples TREATED with the acorn extract.

The evolution of hardness is displayed in **Figure 2B**. There were no differences ($p > 0.05$) in this parameter after cooking and chilling between TREATED and CONTROL samples irrespective of packaging type. However, an increase in hardness was observed in CONTROL samples during the subsequent chilled storage and reheating promoted a decrease in hardness of chicken patties formulated with acorn extract. Interestingly, RTE chicken patties formulated with acorn extract had similar texture properties after reheating that those displayed by freshly made cooked patties. Previous authors have attributed to protein oxidation the texture changes occurred in processed meats that typically involve increases in hardness without any apparent loss of moisture (Ganhão et al., 2010a; Utrera, Parra, & Estévez, 2014). It is hence reasonable to consider that the antioxidant effect of the acorn extract on the chicken proteins could have protected RTE patties from the texture changes occurred in the CONTROL patties.

3.4 Volatiles in RTE chicken patties

The analysis of hexanal (Figure 3A), 1-octen-3-ol (Figure 3B) and deca-2,4-dienal (Figure 3C) was used to evaluate the effect of acorn extract and packaging system on the formation of lipid-derived volatiles with potential odor effects on RTE chicken patties. The evolution of TBARS was similar to that of the hexanal as a marked increase in this aldehyde was noted after cooking and at all subsequent stages (i.e. chilling and reheating) for CONTROL samples. This highlights the role of hexanal as indicator of lipid oxidation in meat systems (Pegg & Shahidi, 2012). The other selected volatiles, 1-octen-3-ol and deca-2,4-dienal, followed a similar behavior while in this case, reheating clearly boosted the formation of these compounds. Both are regarded as odor-active compounds and the alkadienals, in particular are known for imparting

rancid and off-flavors in cooked meats (Estévez et al., 2003). No significant effect of packaging system was observed for the formation of lipid-derived volatiles with the exception of deca-2,4-dienal in reheated CONTROL samples. Conversely, there were significant and considerably notable differences ($p < 0.001$) between TREATED and CONTROL samples for the levels of these lipid-derived volatiles at all processing stages. Hexanal was found at a basal level during the entire processing of TREATED patties with that level being similar to that found in raw patties. Similar results were also found for 1-octen-3-ol while deca-2,4-dienal was not detected in TREATED samples at any of the stages of processing. Together with TBARS results previously reported, these data may substantiate the odor deterioration of RTE chicken patties during storage and processing and the effectiveness of the acorn extract in preventing these unpleasant changes. There is a linear correlation between sensory acceptability, TBARS content and specific volatile compounds formation (Pegg & Shahidi, 2012).

3.5 Color and odor liking of RTE chicken patties

The color liking of the chicken patties was assessed in precooked samples (**Figure 4A**) to analyze the impact of the acorn extract on the color of the freshly made patties. Significant differences ($p < 0.05$) between TREATED and CONTROL samples were found. The scores given by the panelists to the samples were higher in chicken patties formulated with acorn extract. According to the results, the scores provided by the panelists to the treated samples ranged from “like slightly” to “neither like nor disliked” whereas untreated chicken patties had scores ranged from “moderately disliked” to “disliked slightly”.

The odor liking of RTE chicken patties was assessed in cooked, chilled and in samples ready to be consumed (after reheating) and data is shown in **Figure 4B**. As expected, significant differences were found between TREATED and CONTROL samples over the processing stages. Samples formulated with acorn extract received significantly higher sensory scores compared to the CONTROL counterparts. The scores provided by the panelists to the control samples after precooking ranged from “neither like nor disliked” to “like slightly”. The chilled storage promoted a decrease in odor liking of untreated samples, which reached from “moderately disliked” to “disliked slightly” and remained the same after reheating procedure. Chicken patties formulated with acorn extract had the best scores for odor liking, which reached from “like slightly” to “moderately liked” and remained sensory acceptable after chilled and microwave reheating.

CONCLUSIONS

After cooking, ready-to-eat chicken patties suffer intense quality deterioration reflected in high oxidation rates, color and texture deterioration and increasing consumer displeasure. The addition of acorn extract in these products is a feasible and highly effective strategy to inhibit the oxidation-driven changes and preserve the quality of reheated samples as if there were freshly cooked. Based on these results, the use of acorn extract on the chicken patties is more effective than oxygen reduction in MAP in the control of oxidative reactions in a RTE chicken product. In fact, the addition of our extract makes the packaging system irrelevant in terms of quality preservation. However, a more restricted oxygen concentration in MAP samples or even a vacuum package may be also tested in future experiments.

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FIGURES CAPTIONS

Figure 1. Lipid (A) and protein (B) oxidation in raw, cooked, chilled and reheated chicken patties with (TREATED) and without (CONTROL) added oak nut phenolic extract.

Different letters on top of bars denote significant differences ($p < 0.05$) between processing stages and packaging methods (N-OX: normal oxygen concentration; LOW-OX: low-oxygen modified atmosphere) within CONTROL and TREATED samples. Asterisks denote significant differences between CONTROL and TREATED samples at each processing stage; ***: $p < 0.001$; ns: no significant differences ($p > 0.05$).

Figure 2. Evolution of redness (A) and hardness (B) in raw, cooked, chilled and reheated chicken patties with (TREATED) and without (CONTROL) added acorn phenolic extract.

Different letters on top of bars denote significant differences ($p < 0.05$) between processing stages and packaging methods (N-OX: normal oxygen concentration; LOW-OX: low-oxygen modified atmosphere) within CONTROL and TREATED samples. Asterisks denote significant differences between CONTROL and TREATED samples at each processing stage; *: $p < 0.05$; ***: $p < 0.001$.

Figure 3. Evolution of hexanal (A), 1-octen-3-ol (B) and deca-2,4-dienal (C) in raw, cooked, chilled and reheated chicken patties with (TREATED) and without (CONTROL) added acorn phenolic extract.

Different letters on top of bars denote significant differences ($p < 0.05$) between processing stages and packaging methods (N-OX: normal oxygen concentration; LOW-OX: low-oxygen modified atmosphere) within CONTROL and TREATED samples. Asterisks denote significant differences between CONTROL and TREATED

samples at each processing stage; *: $p < 0.05$; ***: $p < 0.001$; ns: no significant differences ($p > 0.05$).

Figure 4. Color liking of cooked chicken patties (A) and odor liking (B) of cooked, chilled and reheated chicken patties with (TREATED) and without (CONTROL) added acorn phenolic extract.

Different letters on top of bars denote significant differences ($p < 0.05$) between processing stages and packaging methods (N-OX: normal oxygen concentration; LOW-OX: low-oxygen modified atmosphere) within CONTROL and TREATED samples. Asterisks denote significant differences between CONTROL and TREATED samples at each processing stage; ***: $p < 0.001$

Table 1. Experimental setting: group of samples as affected by the treatment with acorn extract and processing stage

Processing Stage	Treatment with acorn extract			
	CONTROL (n=24)		TREATED (n=24)	
Raw	CONTROL (n=4)		TREATED (n=4)	
Cooked	CONTROL (n=4)		TREATED (n=4)	
Cooked & Chilled	NORMAL-OX (n=4)	LOW-OX (n=4)	NORMAL-OX (n=4)	LOW-OX (n=4)
Cooked & Chilled & Reheated	NORMAL-OX (n=4)	LOW-OX (n=4)	NORMAL-OX (n=4)	LOW-OX (n=4)

Table 2. Chemical composition and instrumental color (means $\pm$ SD) of raw and pre-cooked chicken patties with (TREATED) and without (CONTROL) added oak nut phenolic extract.

	Raw		Cooked		p ^A		
	CONTROL	TREATED	CONTROL	TREATED	PC	PT	PC x PT
Moisture ^B	74.43a $\pm$ 0.31	74.52a $\pm$ 0.31	66.32b $\pm$ 1.06	66.75b $\pm$ 1.80	***	ns	ns
Protein ^B	15.26b $\pm$ 0.53	14.60b $\pm$ 0.42	19.19a $\pm$ 0.29	19.36a $\pm$ 0.14	***	ns	ns
Lipid ^B	7.03b $\pm$ 0.98	7.51b $\pm$ 0.45	9.57a $\pm$ 0.53	9.96a $\pm$ 0.35	***	ns	ns
Σ SFA ^C	34.73 $\pm$ 0.29	34.31 $\pm$ 0.77	34.59 $\pm$ 1.10	35.72 $\pm$ 1.18	ns	ns	ns
Σ MUFA ^C	39.92 $\pm$ 0.18	40.08 $\pm$ 0.37	39.88 $\pm$ 1.10	39.75 $\pm$ 1.10	ns	ns	ns
Σ PUFA ^C	25.35 $\pm$ 0.15	25.61 $\pm$ 0.40	25.52 $\pm$ 0.40	24.53 $\pm$ 1.15	ns	ns	ns
L*	71.17a $\pm$ 0.47	70.43a $\pm$ 0.41	66.81b $\pm$ 0.74	61.17c $\pm$ 1.11	***	***	***
a*	9.92b $\pm$ 0.34	10.61a $\pm$ 0.36	5.09d $\pm$ 0.90	7.93c $\pm$ 0.46	***	**	**
b*	13.23c $\pm$ 0.36	12.74d $\pm$ 0.37	20.19a $\pm$ 0.72	18.13b $\pm$ 0.41	***	**	*
chroma	16.53b $\pm$ 0.46	16.58b $\pm$ 0.51	20.84a $\pm$ 0.61	19.79a $\pm$ 0.49	***	ns	ns
hue	53.16c $\pm$ 0.60	50.25d $\pm$ 0.31	75.85a $\pm$ 2.70	66.43b $\pm$ 1.06	***	***	***

^A Statistical significance in two-way ANOVA test; PC: Effect of cooking; PT: Effect of treatment with phenolic extract.

^B Data expressed as g/100 g sample.

^C Data expressed as percentage of total fatty acids analyzed.

Different letters denote significant differences ($p < 0.05$) between treatments.

Figure 1.

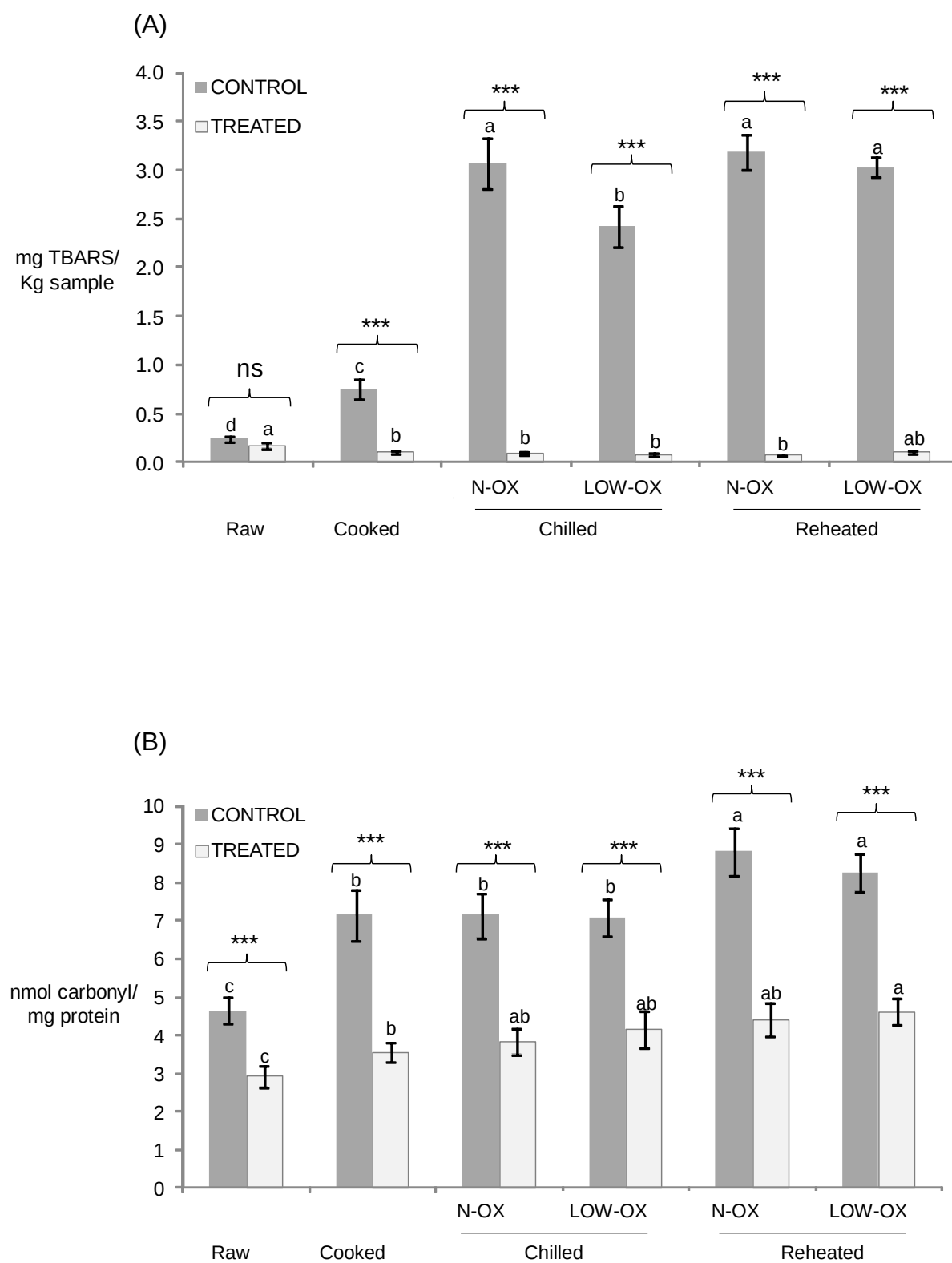


Figure 2.

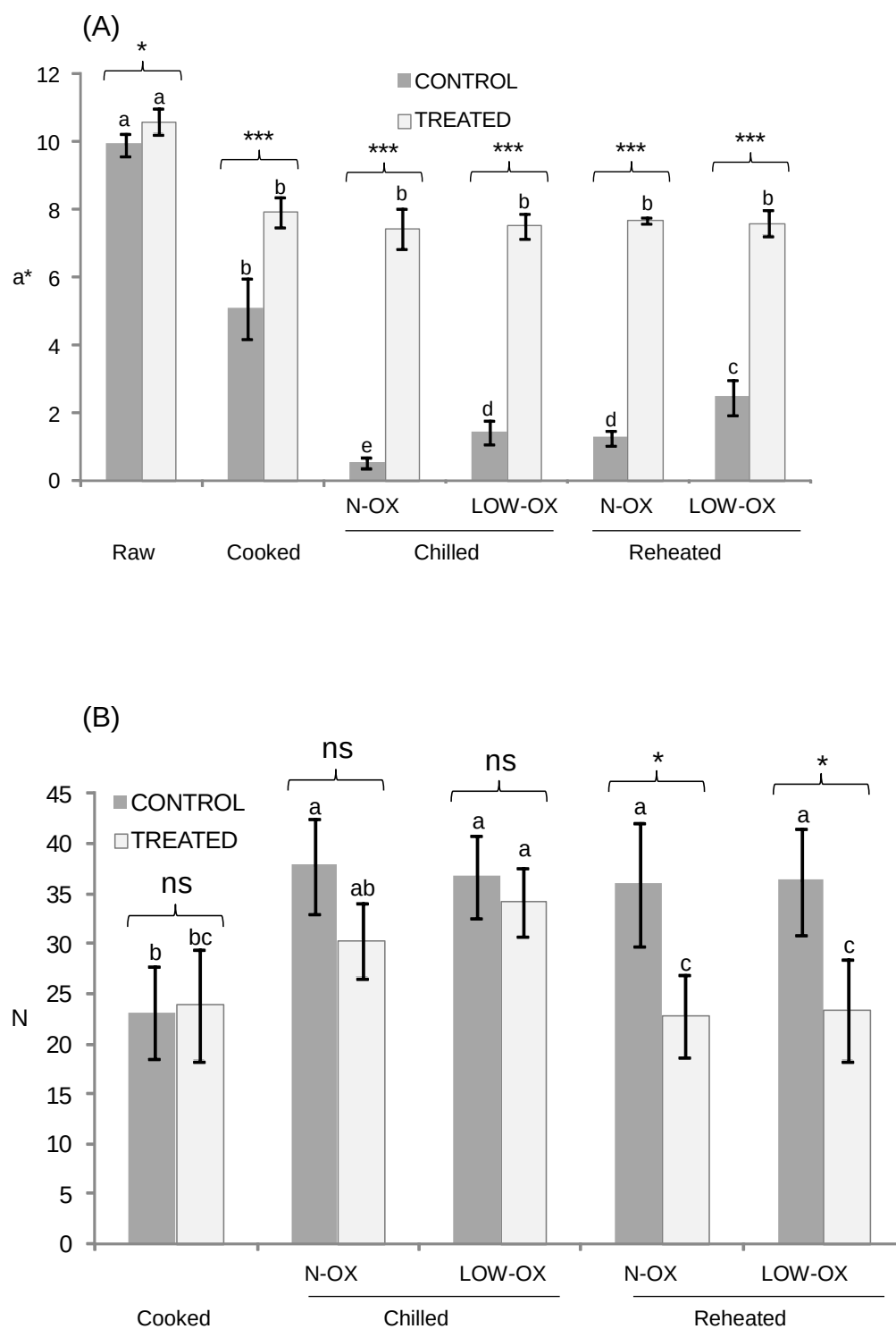


Figure 3.

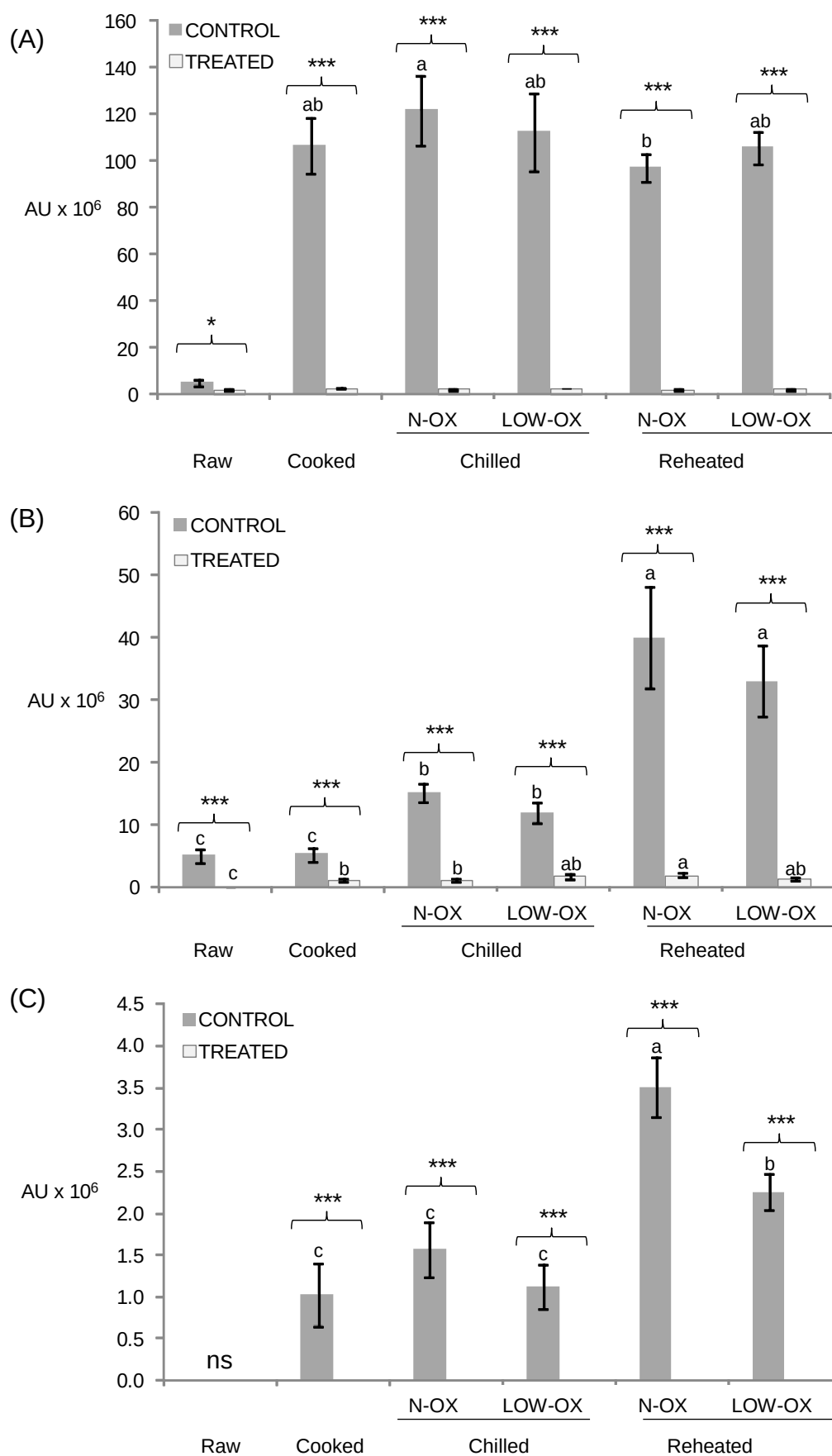
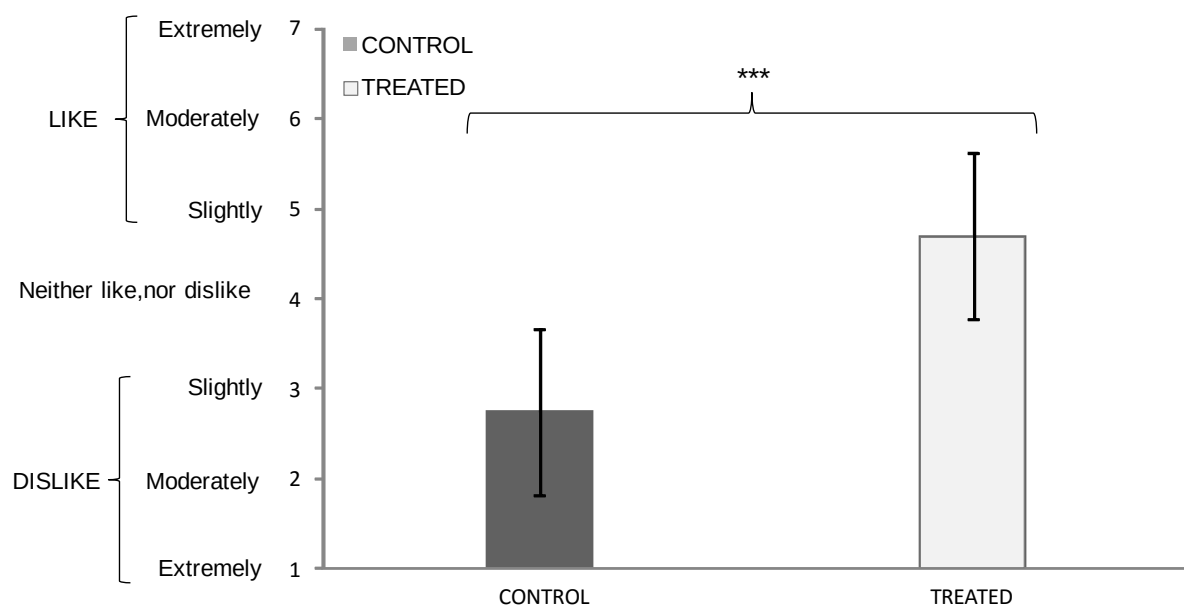
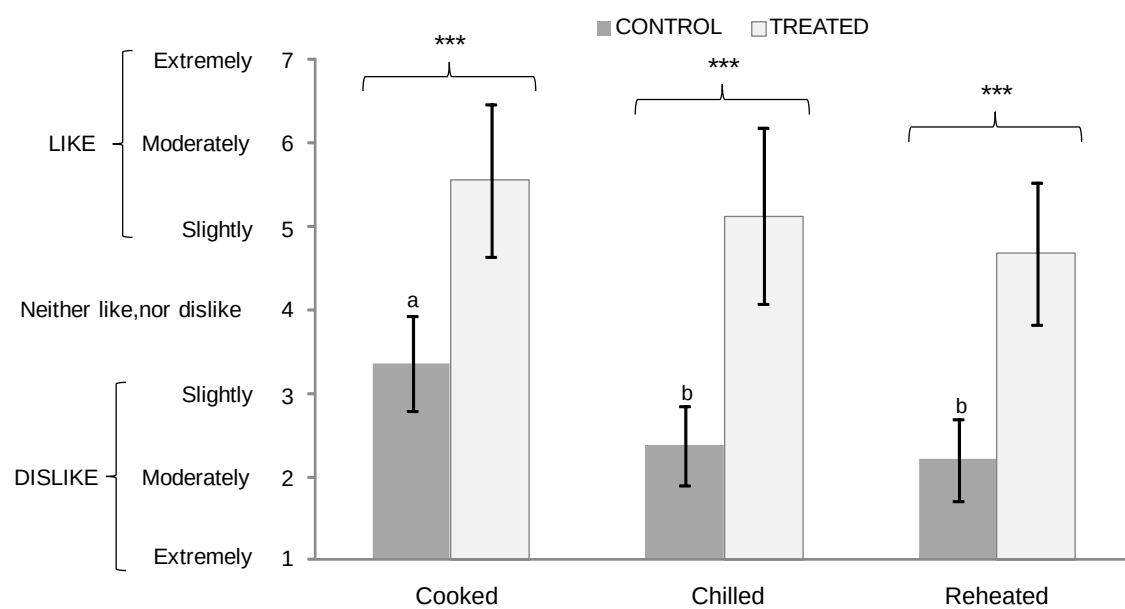


Figure 4.

(A)



(B)



5 CONCLUSÕES GERAIS

De acordo com os experimentos realizados pôde-se concluir que:

- a) Nos emulsionados tipo hambúrguer de frango prontos para consumo, o armazenamento e seu tempo de duração tem influência direta sobre sua deterioração química;
- b) A escolha do tipo de cozimento utilizado nos emulsionados tipo hambúrguer de frango influencia o desenvolvimento da oxidação lipídica, entretanto a deterioração do odor foi semelhante independentemente do método de cozimento aplicado;
- c) A oxidação das proteínas foi maior nos emulsionados tipo hambúrguer de frango cozidos pelo método em água quente, mas durante as etapas de processamento ocorreram modificações consideráveis nos hambúrgueres cozidos pelos métodos em forno e chapa;
- d) A oxidação proteica nos emulsionados tipo hambúrguer de frango prontos para consumo parece estar relacionada com a perda da digestibilidade das proteínas presentes, e em certa medida, com as modificações de dureza;
- e) O uso de extrato vegetal nos emulsionados tipo hambúrguer de frango prontos para consumo, com potencial antioxidante, reduziu os processos oxidativos lipídicos e proteicos;
- f) A aplicação de extrato vegetal, com potencial antioxidante, foi mais eficiente no controle das oxidações lipídica e proteica do que o uso de embalagem com atmosfera modificada;
- g) O uso de extrato vegetal como antioxidante natural em emulsionado tipo hambúrguer de frango pronto para consumo parece ser viável, entretanto é necessário a realização de mais estudos que garantam com segurança que o consumo, por humanos, deste componente não possui consequências indesejáveis a saúde.

ANEXOS

ANEXO A – Comprovante de submissão para o Artigo II.

Elsevier Editorial System(tm) for Food Chemistry

Manuscript Draft

Manuscript Number:

Title: Oxidative degradation of essential amino acids and impaired in vitro protein digestibility in ready-to-eat (RTE) chicken patties

Article Type: Research Article (max 7,500 words)

Keywords: Convenience food; Protein oxidation; Thiols; Tryptophan; Carbonyls; Cross-linking; Hardness

Corresponding Author: Mr. Mario Estevez, Ph.D.

Corresponding Author's Institution: University of Extremadura

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Order of Authors: Valquiria Ferreira; David Morcuende; Marta Madruga; Silvia Lopez; Fabio Silva; Mario Estevez, Ph.D.

Abstract: The oxidative damage to proteins during processing of ready-to eat chicken patties and its impact on the nutritional value and texture properties of the product, were assessed. The effect of pre-cooking techniques (boiling, roasting and grilling), and microwave reheating (600 mW/1min) after 7 and 14 days of cold storage (+4°C) were studied. Cooking methods had an effect ($p < 0.05$) on most protein oxidation parameters, including protein depletion of tryptophan, thiols, protein carbonylation and formation of crosslinks. Overall, the succeeding chilled storage led to an aggravation of the oxidative damage which occurred along with hardness increase and impaired digestibility. Microwave reheating had contradictory effects on protein oxidation parameters. An increase in protein carbonylation occurred along with a cleavage of disulphide bonds that would explain a decrease in hardness. The present study illustrates to which extent the oxidative damage to meat proteins affects protein quality and nutritional value of a severely processed meat product. The impact of various assorted pre-cooked procedures on these events is also discussed.

ANEXO B - Comprovante de submissão para o Artigo III.

Elsevier Editorial System (tm) for Food Chemistry

Manuscript Draft

Manuscript Number:

Title: Phytochemicals from acorn (*Quercus ilex* L.) inhibit oxidation and enhance consumer liking of ready-to-eat chicken patties irrespective of packaging atmosphere

Article Type: Research Article (max 7,500 words)

Keywords: convenience foods; phytochemicals; lipid oxidation; protein oxidation; MAP

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Order of Authors: Valquiria Ferreira; Silvia Hernández-López; David Morcuende; Marta S Madruga; Fabio A Silva; Mario Estevez, Ph.D.

Abstract: This study evaluated the impact of a phenolic-rich acorn extract (200 ppm GAE) and the packaging system (low-oxygen modified atmosphere; 5% vs. normal-oxygen; 21%) on lipid and protein oxidation and consumers liking of the ready-to-eat chicken patties. Samples were subjected to cooking (electric oven, 170°C/16min), cold storage (14 days at 4°C) and reheating (microwave, 600mW/1min). Samples treated with acorn extract kept TBARS numbers and lipid-derived volatiles at basal levels throughout the whole processing irrespective of the packaging type. Consistently, treated patties had lower protein carbonyls than to control ones. The acorn extract also controlled color and texture deterioration during chilled storage and reheating and improved the color and odor liking of the products. Formulating with acorn extract is a feasible strategy to inhibit the oxidation-driven changes and preserve the quality of reheated samples as if there were freshly cooked. The packaging system was irrelevant in terms of quality preservation