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CÉSAR AQUILES LÁZARO DE LA TORRE

UTILIZAÇÃO DA CROMATOGRAFIA LÍQUIDA DE ALTA EFICIÊNCIA NA DETERMINAÇÃO DE AMINAS BIOGÊNICAS COMO FERRAMENTA PARA A AVALIAÇÃO DA QUALIDADE CARNE DE AVES

NITERÓI
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Tese apresentada ao Programa de Pós-Graduação em Medicina Veterinária da Universidade Federal Fluminense, Área de Concentração: Higiene Veterinária e Processamento Tecnológico de Produtos de Origem Animal, como requisito parcial para obtenção de Grau de Doutor.

Orientador: Prof. Dr. Robson Maia Franco

Co-Orientador: Prof. Dr. Carlos Adam Conte Junior

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A mis amados padres Bernardina e Aquiles, que aun en la distancia siempre estuvieron presentes en todos los logros conseguidos.

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"Es muy común recordar que alguien nos debe agradecimiento, pero es más común no pensar en quienes le debemos nuestra propia gratitud"

Johann Wolfgang Goethe

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RESUMO

As aminas biogênicas (AB) são produzidas principalmente pela descarboxilação de aminoácidos específicos de matrizes alimentares, entre estas, carne de aves. Estas substâncias podem ser utilizadas como indicadores de deterioração em diversos alimentos. A Cromatografia Liquida de Alta Eficiência (CLAE) é uma técnica altamente sensível e confiável que vem sendo utilizada na determinação e quantificação de AB em diversos alimentos. O presente trabalho foi dividido em três partes: (1) Validar a metodologia do CLAE para identificar e quantificar aminas biogênicas em carne de frango (Artigo I); os resultados determinaram que a extração das amostras com ácido perclórico a 5% seguido da derivatização com cloreto de benzoila permitiu identificar e quantificar as aminas avaliadas por CLAE com sistema isocrático e detector UV; além disso, evidenciaram-se resultados confiáveis nos parâmetros de validação. (2) Correlacionar os valores das AB com o crescimento bacteriano e parâmetros bioquímicos em cinco tipos de carne de aves (Artigo II e III); os resultados evidenciaram que as aves apresentaram uma tendência similar para o crescimento de Baterias Heterotróficas Aeróbias Mesófilas (BHAM) e Enterobacteriaceae (EM), enquanto o perfil de AB depende do tipo de ave, sendo que tiramina, putrescina e cadaverina parecem ser os parâmetros que melhor explicam a classificação das carnes. Observou-se um incremento gradativo nos valores de pH durante a estocagem em todas as carnes enquanto os valores de TBARS se incrementaram só no primeiro dia ficando estáveis até o final do experimento. (3) Avaliar o efeito do tratamento com luz ultravioleta C (UV-C) nos valores das AB e outros parâmetros utilizados como indicadores de deterioração (Artigo IV). Após a aplicação 90 e 120 segundos da UV-C (0,62, 1,13 e 1,95 mW/cm²) encontrou-se a redução na faixa de 0,33 e 0,60 Log UFC/g de 5 diferentes espécies de *Salmonella* inoculadas em peitos de frango. No tempo de estocagem as exposições da UV-C (1,13 mW/cm² e 1,95 mW/cm²) por 90 segundos diminuiu a carga inicial e retardou as fases de latência de BHAM e EM, incrementou os valores de AB se manteve estáveis os teores de amarelo (b*) até o final do experimento. Baseado nos dados obtidos nas três partes do trabalho, conclui-se que o método proposto foi adequado para a identificação e quantificação simultânea das AB em carne de frango. Tiramina, cadaverina e putrescina podem ser consideradas como critérios de discriminação das aves avaliadas e a cadaverina e putrescina junto com o pH podem ser considerados como indicadores de deterioração. A UV-C teve uma baixa eficiência na redução das espécies de *Salmonella* sem alterar significativamente os parâmetros físico-químicos avaliados. Contudo, as AB parecem não serem os parâmetros mais adequados para usar como indicador de deterioração nestas carnes e sugere-se estudos complementares da UV-C para aprimorar sua aplicação na melhora da qualidade bacteriológica na carne de frango embalada.

Palavras chave: aminas, aves, carne, CLAE, deterioração, UV-C.

ABSTRACT

Biogenic amines (BA) are formed mainly by the decarboxylation of specific amino acids present in matrix food, such as chicken meat. These substances can be used as deterioration indicators in many foods. The High Performance Liquid Chromatography (HPLC) is a sensitive and trusty technique used to detected and quantified BA in different foods. The present work was divided in three parts: (1) Validated a HPLC methodology to identify and quantify biogenic amines (BA) in chicken meat (Paper I); the results determinate that samples extracted with perchloric acid (5%) and derivatized with benzoyl chloride allowed the identification and quantification of BA studied in HPLC with isocratic elution coupled with a UV detector; in the same way, the method showed trusty results for validation parameters. (2) Correlated values of BA with bacterial growth and biochemical parameters in five poultry meats (Paper II and III); the results showed the same growth trend to total aerobic mesophilic bacteria (TAMB) and Enterobacteriaceae (EN) in chicken meats but the BA profile depending on the poultry species. It is suggest that tyramine, putrescine and cadaverine seem to be the parameters that best explains the classification of poultry meats. A gradual increment of pH values in all poultry meats during the storage time and an increment of TBARS values only in the first days of storage remaining stables to the end of experiment were observed. (3) Evaluated the effect of ultraviolet C light (UV-C) in BA and other parameters used as a deterioration indicators (Paper IV). Application of 90 and 120 seconds the UV-C (0.62, 1.13 and 1.95 mW/cm^2) showed a reduction of *Salmonella* species in the range of 0.33 and 0.60 Log CUF/g in inoculated chicken breast. In the storage time both UV-C intensities (1.13 and 1.95 mW/cm^2) for 90 seconds reduced and retarded the bacterial growth curve of TAMB and EN; increased the BA values and yellowness (b*) remained stables to the end of experiment. In conclusion, the proposed method was suitable to simultaneous detection and quantification of BA in chicken meat. Tyramine, putrescine and cadaverine, can be considered to be discrimination criteria among poultry meats and pH, cadaverine and putrescine could be used as a deterioration indicators. UV-C was a low efficiency in reduction of *Salmonella* species in chicken meat without significant changes in the physicochemical parameters studied. The BA are not suitable like indicator of deterioration in these meats and it is suggest complementary studies using UV-C to improve the bacteriological quality in packed chicken meat.

Keywords amines, deterioration, HPLC, meat, poultry, UV-C

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1 INTRODUÇÃO

Na atualidade o Brasil é o segundo maior produtor mundial de carne de frango, abaixo apenas dos Estados Unidos. Este crescimento foi impulsionado principalmente pelo aumento do consumo interno e pela expansão nas exportações (ABEF, 2011). Além disso, tem se observado grande interesse dos consumidores em diferentes carnes de ave como frangos orgânicos e caipiras, pato e codorna devido a seus atributos sensoriais e por serem consideradas mais saudáveis que outras carnes; por este motivo a indústria avícola tem grande interesse pela produção e comercialização destas aves (BRASIL, 1999; 2008).

Devido ao incremento da demanda mundial por carne de aves, os fornecedores são obrigados a implantar controles minuciosos para garantir a qualidade deste produto. A carne é especialmente suscetível à degradação proteica e a determinação das substâncias originadas na degradação podem ser utilizadas como indicadoras da qualidade (DADÁKOVÁ et al., 2009). Indicadores como carga microbiana, modificações no pH, substâncias reativas ao ácido 2-tiobarbitúrico e cor são atributos utilizados frequentemente na avaliação da qualidade da carne. Nesse contexto, a pesquisa de aminas biogênicas contribui com uma correta avaliação da qualidade. As aminas biogênicas são substâncias de baixo peso molecular, formadas principalmente pela descarboxilação de aminoácidos específicos por ação microbiana, assim sua presença na matriz alimentar tem correlação direta com a composição de aminoácidos, microbiota presente, temperatura de estocagem, tempo de maturação, embalagem, entre outros fatores (SUZZI; GARDINI, 2003).

Avaliar a produção e concentração de aminas biogênicas em alimentos têm expressiva relevância na prevenção de intoxicações alimentares, no acompanhamento nos processos de produção de diversos alimentos e na avaliação da qualidade. Segundo a Autoridade Europeia de Segurança Alimentar (EFSA), os métodos baseados na utilização da Cromatografia Líquida de Alta Eficiência (CLAE) são técnicas confiáveis e com alta sensibilidade na detecção e quantificação simultânea de diversas aminas biogênicas (EFSA, 2011). Contudo, a implementação de uma nova metodologia destinada a análises deve ser devidamente validada considerando-se de forma objetiva sua aplicabilidade para uma utilidade prevista (EURACHEM, 1998; ISO, 1999).

As variações qualitativas e quantitativas das aminas biogênicas durante o tempo de estocagem têm sido estudadas em diversos produtos, contudo ainda não se conhece como seria seu comportamento após de certos processos utilizados para reduzir a carga microbiana. A luz ultravioleta tipo C (UV-C) desencadeia reações químicas que atuam nas ligações das moléculas do DNA dos microrganismos, impedindo o desenvolvimento microbiano. Tratamentos com UV-C podem aumentar a validade comercial dos alimentos sem interferir nos parâmetros de qualidade, constituindo uma alternativa para o controle microbiano (KOUTCHMA et al., 2009).

Em vista do apreciado, objetivou-se no presente trabalho a implementação e validação de uma metodologia para identificar e quantificar aminas biogênicas utilizando a cromatografia liquida de alta eficiência em carne de aves, relacionar os valores das principais aminas com o crescimento bacteriano e outros parâmetros bioquímicos e finalmente avaliar as mudanças produzidas pela luz UV-C nos valores das aminas biogênicas e outros parâmetros bacterianos e bioquímicos utilizados na avaliação da qualidade carne de aves.

2 FUNDAMENTAÇÃO TEÓRICA

2.1 CARNE DE AVES COMERCIALIZADAS NO BRASIL

A produção de carne de frango chegou a 12.230 milhões de toneladas em 2010, com um crescimento de 11,38% em relação a 2009, quando foram produzidas 10.980 milhões de toneladas. Com este desempenho o Brasil se aproximou da China, hoje o segundo maior produtor mundial, cuja produção de 2010 teria somado 12.550 milhões de toneladas, abaixo apenas dos Estados Unidos, com 16.648 milhões de toneladas, conforme projeções do Departamento de Agricultura dos EUA (ABEF, 2011).

O crescimento em 2010 foi impulsionado, principalmente, pelo aumento de consumo de carne de frango e pela expansão de 5,1% nas exportações. Do volume total de frangos produzido pelo país, 69% foi destinado ao consumo interno, e 31% para exportações. Com isto, o consumo *per capita* de carne de frango foi de 44 quilos em 2010. Os embarques de 3,819 milhões de toneladas em 2010 representaram o aumento de 5,1% em relação a 2009, em novo recorde histórico para a carne de frango, principal produto das exportações avícolas brasileiras. No caso da receita cambial, de US$ 6.808 milhões, o incremento foi de 17%. O preço médio das vendas brasileiras foi de US$ 1.782 a tonelada, com o aumento de 11,4% (ABEF, 2011).

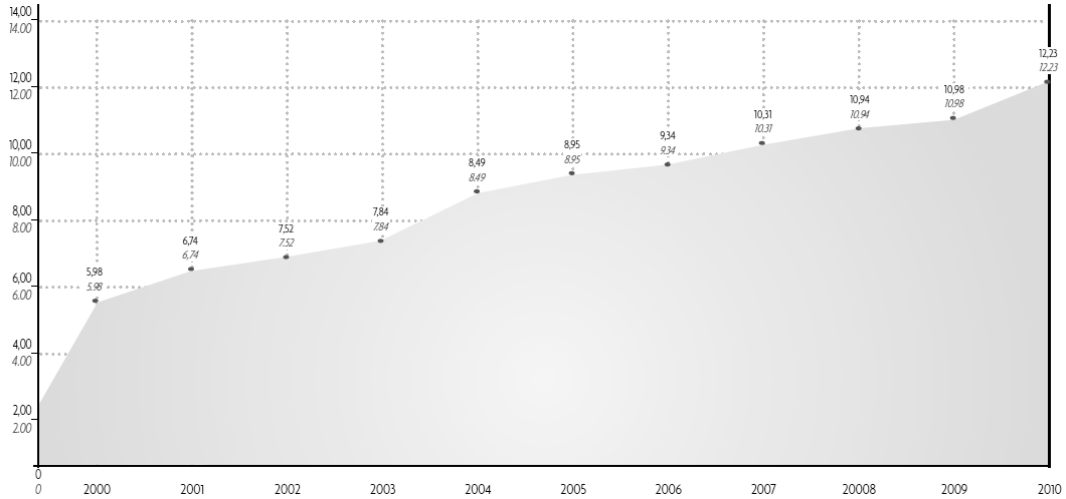


Figura 1. **Produção Brasileira de carne de frango (mil ton)**

Fonte: ABEF 2011.

No setor avícola, na tentativa de satisfazer novas tendências de consumo, vem se buscando alternativas para atender rapidamente a demanda crescente por carnes alternativas à carne de frango convencional, que apresentem um diferencial qualitativo. Dentre os produtos pode-se destacar a produção de frangos orgânicos no qual são adotadas práticas de produção menos agressivas, que aproveita ao máximo o uso de recursos naturais, objetivando-se a auto-sustentação, com a produção de alimentos saudáveis, de elevado valor nutricional, preservando-se a biodiversidade em que se insere esse sistema produtivo, sendo fundamental a redução do emprego dos insumos artificiais, como aditivos e/ou estimulantes, respeitando as normas de bem-estar animal, dispondo-se de instalações funcionais e confortáveis, com alto nível higiênico, em todo o processo criatório. O controle de enfermidades é realizado pela adoção de medidas preventivas, respeitando-se as normas de saúde pública vigentes (BRASIL, 2008).

Outros tipos de produtos alternativos como o frango caipira, proveniente de um sistema de criação semi-intensivo que se tenta dar às aves certo grau de liberdade, disponibilizando um aviário, onde ficam os comedouros, bebedouros e ninhos, mas também lhes dando a opção de ter acesso a uma área livre para pastagem e recreação (BRASIL, 1999); além de patos e codornas também tem mostrado ser uma boa alternativa para os produtores avícolas (CARIONI et al., 2001; OLIVEIRA et al., 2005).

2.2 AMINAS BIOGÊNICAS

2.2.1 Definição

As aminas bioativas ou biologicamente ativas são bases orgânicas alifáticas, alicíclicas ou heterocíclicas de baixo peso molecular. São, também, definidas como compostos nitrogenados, em que um, dois ou três átomos de hidrogênio da amônia foram substituídos por grupos alquila ou arila, como a histamina, que é uma molécula hidrofílica, apresentando um anel imidazólico e um grupo amino conectados por dois grupos metileno. As aminas são formadas por processos bioquímicos e participam de funções metabólicas e fisiológicas importantes nos organismos vivos, desempenhando diversas atividades biológicas. São encontradas em alimentos de origem animal, vegetal, bem como em alimentos fermentados (HALÁSZ et al., 1994).

As aminas bioativas podem ser classificadas em função do número de grupamentos amina na molécula, da estrutura química (Figura 2), da via biossintética e da função que exercem. Quanto ao número de grupamentos amina na molécula, se classificam em monoaminas como tiramina e feniletilamina, diaminas como histamina, triptamina, serotonina, putrescina e cadaverina e poliaminas como espermidina, espermina e agmatina (Figura 3) (RUIZ-CAPILLAS; JIMÉNEZ-COLMENERO, 2004).

Em relação à estrutura química, as aminas podem ser classificadas em alifáticas (putrescina, cadaverina, espermidina, espermina e agmatina), aromáticas (tiramina e feniletilamina) e heterocíclicas (histamina e triptamina). Ainda, em relação à estrutura química, podem ser classificadas em catecolaminas (dopamina, noradrenalina e adrenalina), indolaminas (serotonina) e como imidazolaminas (histamina).

Quanto à via biossintética, as aminas se classificam em naturais, que são formadas durante a biossíntese "*in situ*" a partir de uma molécula mais simples, à medida que são requeridas (espermina e espermidina), ou podem estar armazenadas nos mastócitos e basófilos (histamina). Contudo, as aminas biogênicas são formadas por reações de descarboxilação conduzidas por descarboxilases bacterianas, sendo esta a principal via de formação de aminas nos alimentos (histamina, serotonina, tiramina, feniletilamina, triptamina, putrescina, cadaverina e agmatina) (RUIZ-CAPILLAS; JIMÉNEZ-COLMENERO, 2004; HALÁSZ et al., 1994)

2.2.2 Fatores que influenciam a formação

A formação de aminas biogênicas nos alimentos está condicionada à disponibilidade de aminoácidos livres, presença de microrganismos descarboxilase positivos e, também, às condições favoráveis para o crescimento bacteriano, síntese e ação de enzimas descarboxilases (SHALABY, 1996).

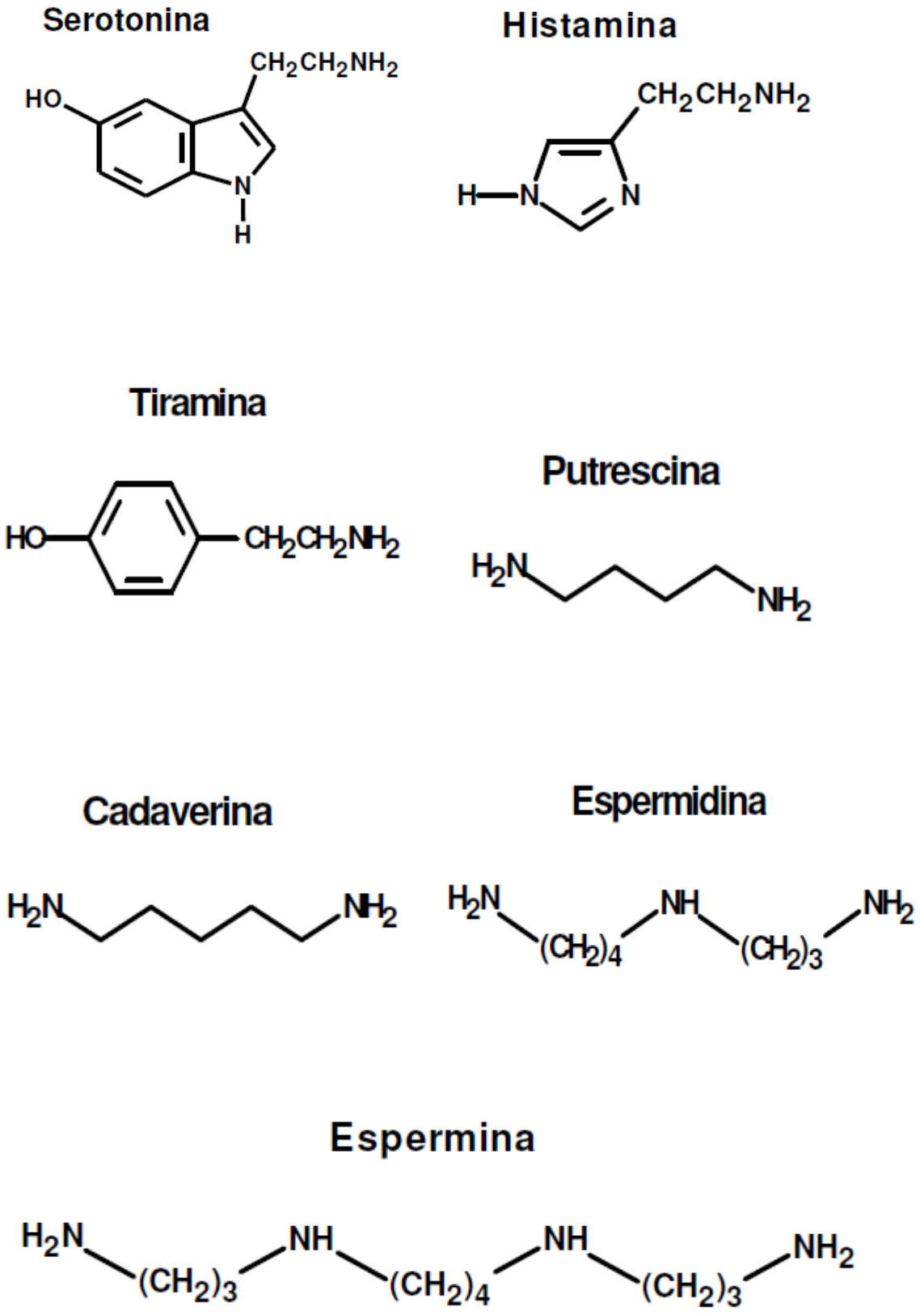


Figura 2. Estrutura química de algumas aminas biogênicas.

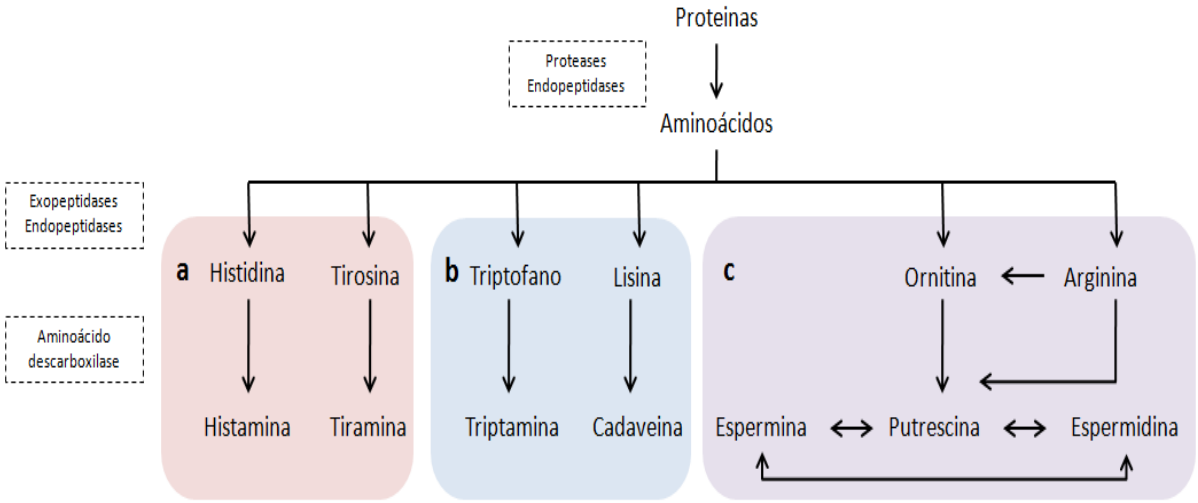


Figura 3. Formação de aminas biogênicas. a) monoaminas, b) diaminas, e c) poliaminas.
Fonte: Ruiz-Capillas; Jiménez-Colmenero (2004).(Modificado)

Os microrganismos com atividade descarboxilase sobre os aminoácidos podem fazer parte da microbiota associada ao alimento, serem introduzidos para obtenção de produtos fermentados, ou ainda por contaminação antes, durante ou depois do processamento. A quantidade e o tipo de aminas nos alimentos em geral, dependem da natureza, origem, etapas de processamento e microrganismos presentes. Dentre os gêneros bacterianos capazes de descarboxilar um ou mais aminoácidos estão incluídos os gêneros: *Bacillus, Citrobacter, Clostridium, Escherichia, Klebsiella, Lactobacillus, Pediococcus, Photobacterium, Proteus, Pseudomonas, Salmonella, Shigella* e *Streptococcus*. Espécies de Enterobacteriaceae produzem histamina, tiramina, putrescina e cadaverina (HALÁSZ et al., 1994).

As aminas em alimentos podem estar naturalmente presentes no produto, ou serem formadas por microrganismos adicionados (culturas iniciadoras) ou contaminantes, introduzidos devido às condições higiênico-sanitárias inadequadas. Assim sendo, podem ser utilizadas como parâmetro ou critério de qualidade, refletindo a má qualidade das matérias-primas utilizadas e/ou das condições higiênico-sanitárias durante a fabricação de certos produtos (HALÁSZ et al., 1994).

Para a formação de aminas biogênicas é necessária a presença de aminoácidos livres (AAL), enzima descarboxilase e condições adequadas. Assim, todos os fatores que influem na produção do substrato (AAL), da enzima e de sua atividade, afetam o tipo e a quantidade de amina biogênica presente nos alimentos. Fatores associados com a matéria-prima (composição da carne, pH, manipulação, etc.) afetam a disponibilidade de AAL, entretanto, a presença da enzima está intimamente ligada à aspectos microbiológicos (espécies e cepas bacterianas e sua fisiologia e metabolismo). Esses fatores são interdependentes e, obviamente, são mais influenciados pelos processos tecnológicos associados com os tipos de produtos cárneos (assado, bife, presunto, reestruturado, triturados, fresco, cozido, defumado, fermentado, etc) e de armazenamento (tempo / temperatura, embalagem, mudanças de temperatura, etc.) A ação combinada desses fatores é determinante nas concentrações finais de aminas biogênicas (RUIZ-CAPILLAS; JIMÉNEZ-COLMENERO, 2004).

2.2.3 Funções biológicas e efeitos toxicológicos

Além de seu papel biológico como fonte de nitrogênio e precursores na síntese de hormônios, alcalóides, ácidos nucléicos e proteínas, as aminas são importantes componentes do aroma de alimentos e precursores potenciais da formação de compostos nitrogenados cancerigenos. Poliaminas (putrescina, espermina e espermidina) são componentes indispensáveis das células, todas as células tem a capacidade de sintetizar poliaminas, as quais também podem ser derivadas de fontes externas como a dieta. Certas classes de aminas, as catecolaminas, indolaminas e histamina, cumprem importantes funções metabólicas em humanos, especialmente no sistema nervoso e no controle da pressão sanguínea. Feniletilamina e tiramina causam aumento na pressão sanguínea; a histamina possui uma função biológica importante, servindo como um mediador primário dos sintomas imediatos percebidos em respostas alérgicas. Putrescina, cadaverina e agmatina têm sido identificadas como potencializadoras da ação tóxica de histamina em humanos (SILLA SANTOS, 1996).

Quadro 1. Efeitos toxicológicos de algumas aminas biogênicas

Amina biogênica	Efeito toxicológico
Histamina	Cefaleia, sudorese, secreção e ardor nasal, facial, erupções cutâneas vermelhas, edema cutâneo (pálpebras), urticária, dificuldade de deglutição, diarreia, desconforto respiratório, bronco espasmo, aumento do débito cardíaco, taquicardia, extra-sístoles, distúrbios de pressão sanguínea.
Tiramina	Dores de cabeça, enxaqueca, transtornos neurológicos, náuseas, vômitos, distúrbios respiratórios, hipertensão.
Putrescina	Aumento do débito cardíaco, taquicardia, hipotensão, efeitos cancerígenos.

Adaptado de Ladero et al. (2010)

A tiramina, a histamina e, em menor grau, a 2-feniletilamina podem desencadear reações adversas quando são consumidas. Devido às suas propriedades vasoativas, a tiramina e a 2-feniletilamina podem conduzir ao aumento da pressão artéria; podendo causar ainda cefaleia, sudorese, vômitos, entre outros sintomas. A histamina pode provocar vasodilatação e subsequente hipotensão, problemas de pele (rubor e prurido), problemas gastrointestinais (diarreia, cólicas, vómitos) e neurológicos (dores de cabeça, tonturas), entre outros (Quadro 1). A histamina tem sido implicada como agente causal em diversos surtos de intoxicação alimentar e pensa-se que pode ter uma toxicidade acrescida quando na presença de outras aminas como a cadaverina, putrescina e tiramina. A agmatina, a espermina, a espermidina ou as aminas terciárias podem ainda reagir com nitritos formando nitrosaminas carcinogênicas que constituem um potencial risco para a saúde humana. (VIDAL-CAROU et al., 2009)

2.2.4 Ocorrência nos alimentos

Todos os alimentos que contém proteínas ou aminoácidos livres são sujeitos a condições que permitem a atividade microbiana e bioquímica que podem ser favoráveis para a produção de aminas biogênicas. A quantidade total das diferentes aminas formadas depende principalmente da natureza do alimento e dos microrganismos presentes; estas substâncias podem ser encontradas em diversos produtos incluindo pescados, carnes, lácteos, vinho, cerveja, vegetais, frutas e chocolate (SILLA SANTOS, 1996; GLÓRIA, 2005; CACCIOPPOLI et al., 2006; SILVA et al., 2011).

Apesar de muitas aminas biogênicas serem encontradas em peixes, apenas histamina, cadaverina, e putrescina, possuem um valor significativo na determinação da qualidade em pescados. Existe uma associação amplamente difundida entre histamina e a intoxicação alimentar por peixes escombrídeos, mas parece ser que outras aminas como putrescina e cadaverina seriam agentes que potencializam a toxicidade da histamina. Contudo, em referencia à deterioração, apenas a cadaverina pode ser um indicador útil na etapa inicial de decomposição em peixes devido a seu incremento significativo (GLÓRIA, 2005; BULUSHI et al., 2009).

Nas carnes recém-abatidas, espermina e espermidina são as principais aminas detectadas por serem de origem endógena. Além de pequenas quantidades de putrescina que podem pontualmente ocorrer, as outras aminas são normalmente indetectáveis e só aparecem sob condições que permitam a atividade bacteriana. Nos produtos cárneos submetidos a tratamento térmico os níveis de espermina e espermidina são, em geral, ligeiramente inferiores aos da carne fresca, sendo este fato atribuído ao efeito da diluição produzida quando a carne magra é misturada com gordura e outros ingredientes incluídos na formulação do produto. Embora as poliaminas sejam consideradas resistentes ao calor, uma pequena redução destes compostos também tem sido relatada durante os tratamentos térmicos. O conteúdo das outras aminas biogênicas nestes produtos é muito mais variável do que o das poliaminas (VIDAL-CAROU et al., 2009). Durante o processo de curado existe muita atividade proteolítica do músculo, principalmente por ação das proteinases; enquanto que na fermentação a proteolise é favorecida pela desnaturação proteica como consequência da acidez, desidratação e ação do sal. Todo isso contribui a modificar a composição do nitrogênio não proteico e a produção de aminoácidos livres (GLÓRIA, 2005)

O período de maturação é um fator crítico que determina o grau de acumulação das aminas biogénicas, especialmente a tiramina. Em contrapartida, uma grande formação de diaminas durante o fabrico, especialmente a cadaverina, tem sido relatada como dependente do tipo de maturação. A maturação rápida permite um crescimento maior das aminas biogénicas em comparação com a maturação lenta. Estes resultados são atribuídos às altas temperaturas aplicadas durante a secagem/cura. Os fenômenos proteolíticos que ocorrem durante o madurecimento

aumentam a concentração dos aminoácidos precursores e correlacionam-se com a formação das aminas biogênicas (VIDAL-CAROU et al., 2009).

Embutidos secos podem ser fermentados espontaneamente por microflora utilizada como iniciadores os quais poderiam produzir quantidades significativas de putrescina e tiramina. A seleção de bactérias iniciadoras que contribuíam na redução da produção dessas aminas. As culturas iniciadoras, principalmente bactérias ácido láticas, reduzem o tempo de fermentação, contudo este grupo de bactérias é associado com a produção de aminas (GLÓRIA, 2005).

2.2.5 Indicadores de qualidade

As aminas biogênicas são de interesse e utilizadas devido à sua relação com a qualidade dos alimentos. DANQUAH et al. (2012) realizaram uma ampla revisão sobre o uso das aminas como indicadoras de qualidade, indicando que soluções aquosas de putrescina e cadaverina conferem um odor característico e perceptível a níveis de 22 e 190 ppm respectivamente. A relação das aminas com o sabor ainda não é bem estabelecida, porém as pesquisas têm sido objetivadas para o uso destas aminas como indicadoras químicas da qualidade dos alimentos. Ainda estes autores relataram que a relação entre as aminas e que a deterioração dos alimentos podem ser utilizadas para estimar um parâmetro conhecido como índice de qualidade química ou Índice de Aminas Biogênicas (IAB) em pescados, sendo utilizadas concentrações de putrescina, cadaverina, histamina, espermina e espermidina, sendo calculada pela seguinte formula:

$$\text{Índice de Aminas Biogênicas (IAB)} = \frac{\text{Histamina + Putrescina + Cadaverina}}{\text{1 + Espermidina + Espermina}}$$

Valores deste índice menores do que 1 significam um produto de boa qualidade, valores entre 1 e 10 são indicadores de produtos de baixa qualidade; contudo, valores superiores a 10 evidenciam deterioração. O IAB também foi utilizado em outros alimentos como a cerveja, mas foram consideradas outras aminas como tiramina, *β*-fenilalanina e agmatina que são mais frequentes neste produto, sendo calculado com a seguinte formula:

$$\text{IAB} = \frac{\text{Histamina + Putrescina + Cadaverina + Tiramina + Triptamina + Fenilalanina}}{\text{1 + Agmatina}}$$

2.2.6 Microrganismos produtores de aminas biogênicas

As enzimas bacterianas descarboxilase são fundamentais na formação de aminas biogênicas, da mesma forma, os microrganismos que produzem estas enzimas são elementos extremadamente importantes (Quadro 2). A quantidade e tipo de aminas formadas nos alimentos vão depender dos microrganismos presentes. Em produtos cárneos fermentados as culturas iniciadoras (geralmente bactérias acido láticas) parecem ser importantes produtoras de aminas, contudo os microrganismos contaminantes, que podem estar presentes no produto por deficiências no processo de elaboração e armazenamento, também podem produzir grandes quantidades de aminas biogênicas (PAPAVERGOU et al., 2012).

Quadro 2. Produção de aminas biogênicas por microrganismos em carne e derivados.

Produto	**Microrganismo**	**Amina biogênica**
Carne bovina	*Pseudomonas*	Putrescina
Carne suina	*Carnobacterium, Lactobacillus curvatus, Lactobacillus plantarum*	Tiramina
Carne suina (5°C)	*Enterobacter cloacae*	Putrescina
	Klebsiela pneumoniae	Cadaverina
Carne bovina, suina e ovina (5°C)	Enterobacteriaceae	Cadaverina
	Bacterias aerobias totais	Putrescina
Carne embalada e nao embalada (bovino, suino e Coelho)	Enterobacteriaceae	Cadaverina
	Pseudomonas	Putrescina
Carne moída e processada	*Enterobacteriacea*	Cadaverina
Carne bovina embalada ao vácuo (1°C)	*Lactobacillus divergens,*	Tiramina
	Lactobacillus carnis	Cadaverina
	Hafnia alvey	Putrescina
	Serratia liquefaciens	
Suino estocado em CO2/aire (2°C)	*Brochothrix thermosphacta*	Cadaverina
	Lactobacilli, Enterobacteriaceae	Putrescina
Embutidos secos	*Lactobacilli*	Histamina
	Carnobacterium, Lactobacillus plantarum	Tiramina
Embutidos fermentados	Baterias ácido láticas	Tiramina
	Enterobacteriaceae	Cadaverina

Adaptado de Ruiz-Capillas; Jiménez-Colmenero (2004).

Enterobacteriaceae também são consideradas importantes na formação de aminas biogênicas devido principalmente a sua elevada atividade descarboxilase. DURLU-ÖZKAYA et al. (2001) avaliaram a capacidade de produzir aminas

biogênicas em 44 cepas de Enterobacteriaceae inoculadas em caldo BHI suplementado com aminoácidos precursores, detectando que histamina pode ser formada por cepas de *Escherichia coli*, *Morganella morganii* e *Proteus mirabilis;* cadaverina é produzida por *Escherichia. coli*, *Escherichia aerogenes* e *Klebsiella pneumoniae* e altas quantidades de putrescina foram produzidas por *Citrobacter freundii, Enterobacter* spp, *Serratia grimesii, Proteus alcalifaciens, Escherichia coli, Escherichia fergusonii, Morganella morganii, Proteus mirabilis, Proteus penneri e Hafnia alvei*.

2.2.7 Controle da formação de aminas biogênicas em alimentos

A formação de aminas biogênicas pode ser controlada com o uso de métodos tradicionais ou métodos emergentes. Entre os métodos tradicionalmente utilizados esta a aplicação de baixas temperaturas. Em temperaturas de refrigeração os níveis de aminas diminuem devido a inibição do crescimento bacteriano e redução da atividade enzimática; devido a este fato, um adequado controle na cadeia de frio pode ser útil na redução das aminas; sendo as temperaturas de congelamento mais efetivas do que as de refrigeração. Os métodos emergentes incluem embalagens de atmosfera modificada, irradiação, alta pressão hidrostática (NAILA et al., 2010).

2.2.8 Métodos para determinar aminas biogênicas

Como citado anteriormente, são dois os motivos principais para se determinar aminas biogênicas nos alimentos, sua potencial toxicidade e a possibilidade de serem utilizadas como indicadores de qualidade. Independentemente da metodologia utilizada existem dois inconvenientes na análise de aminas biogênicas, a complexidade da matriz e as baixas concentrações dos compostos presentes. Devido à presença de componentes que interferem na matriz alimentar, a determinação simultânea de aminas é difícil de ser realizada. Para obtenção de um resultado o mais fidedigno possível, o processo de extração deve ser realizado com uma série de solventes que consigam separar as aminas biogênicas de outros compostos, por tanto utilizam-se ácido clorídrico, ácido tricloroacético, ácido perclórico e éter de petróleo (ÖNAL, 2007).

Existem vários métodos para determinação de aminas biogênicas: Cromatografia em Camada Delgada (CCD), Cromatografia Gasosa (CG), métodos Electroforeticos Capilares (EC) e Cromatografia Líquida de Alta Eficiência (CLAE).

CCD é uma técnica simples é não requer de equipamentos especiais, em contraposição é muito demorada e os resultados são semi-quantitavos. CG não é usualmente aplicado para a determinação de aminas. EC com detecção fluorescente foi utilizado com sucesso, mas devido às aminas não exibirem uma fluorescência alta, elas não podem ser detectadas diretamente. De todas as metodologias a CLAE com derivatização pré ou pós-coluna é a técnica mais utilizada na separação e quantificação de aminas (ÖNAL, 2007).

Devido à baixa volatilidade e falta de cromóforos uma série de reagentes derivatizantes como dansil, benzoil, fluoresceina, *o*-phtalaldeido (OPA), naftaleno-2,3-dicarboxaldeido têm sido utilizados. OPA pode reagir facilmente com aminas primárias depois de 30 segundos em presença de um agente redutor (*N*-acetilcisteina ou 2-mercaptoetanol) mas o derivado não é muito estável. Dansil e benzoil têm sido mais utilizadas por reagirem com aminas primárias e secundárias, serem estáveis, e poderem ser detectados com UV (ÖNAL, 2007).

2.2.9 Uso da quimiometria na análises de aminas biogênicas

A análise de dados é uma parte essencial em todo experimento, sendo univariada quando somente uma variável é medida sistematicamente para várias amostras. Há muito tempo a estatística univariada vem sendo aplicada a problemas químicos, mas a sua utilização tornou-se limitada. Nas últimas décadas, a análise multivariada foi introduzida no tratamento de dados químicos, ganhando rapidamente popularidade e dando origem a uma nova disciplina, denominada de Quimiometria. No modelo estatístico multivariados é considerada a relação entre muitas variáveis analisadas simultaneamente, permitindo a extração de uma quantidade muito maior de informação (MOITA NETO; MOITA, 1998; SENA et al., 2000).

Em alimentos são varias as técnicas estatísticas para uma melhor interpretação e apresentação dos resultados. GENNARO et al. (2003) utilizaram a quimiometria para pesquisar a relação dos níveis de putrescina, cadaverina, histamina e tiramina em queijos correlacionando-os com três parâmetros de elaboração: pré-tratamento do leite (cru o pasteurizado), a cultura iniciadora (mesofílica ou termofílica) e a temperatura do coalho (quente o frio) e relataram que o processo que produz menos quantidade de aminas é aquele que usa leite pasteurizado, culturas iniciadoras

mesófilas e coalho quente. YAÑEZ et al. (2012) relacionaram as aminas biogênicas com os tipos de vinhos produzidos com uvas tradicionais ou orgânicas e relataram que existe uma classificação das aminas biogênicas baseada no tipo de cultivo de uva utilizada para a produção de vinho sendo que uma maior concentração de aminas foi observada em aqueles que vieram de cultura tradicional.

A redução de variáveis através de critérios objetivos, permitindo a construção de gráficos bidimensionais, contendo maior informação estatística, pode ser conseguida através da análise de componentes principais. Também é possível construir agrupamentos entre as amostras de acordo com suas similaridades, utilizando todas as variáveis disponíveis, e representá-los de maneira bidimensional através de um dendrograma. A análise de componentes principais e de agrupamento hierárquico são técnicas de estatística multivariada complementares que têm grande aceitação na análise de dados químicos (MOITA NETO; MOITA, 1998).

2.2.9.1 Análise de agrupamento hierárquico

A técnica de agrupamento hierárquico é que interliga as amostras por suas associações, produzindo um dendrograma onde as amostras semelhantes, segundo as variáveis escolhidas, são agrupadas entre si. A suposição básica de sua interpretação é esta: quanto menor a distância entre os pontos, maior a semelhança entre as amostras. Os dendrogramas são especialmente úteis na visualização de semelhanças entre amostras ou objetos representados por pontos em espaço com dimensão maior do que três, onde a representação de gráficos convencionais não é possível. Existem muitas maneiras de procurar agrupamentos no espaço n-dimensional. O método matemático mais simples consiste em agrupar os pares de pontos que estão mais próximos, usando a distância euclidiana, e substituí-los por um novo ponto localizado na metade da distância entre os pontos (MILLER; MILLER, 2005; BRERETON, 2007).

Os dendrogramas, portanto, consistem em diagramas que representam a similaridade entre pares de amostras (ou grupos de amostras) numa escala que vai de um (identidade) a zero (nenhuma similaridade). Os dendrogramas são construídos diretamente por todos os programas estatísticos que fazem classificação

dos dados através de agrupamento hierárquico: “Hierarchical Analysis” ou “Cluster Analysis”

2.2.9.2 Análise de componentes principais

A análise de componentes principais consiste essencialmente em reescrever as coordenadas das amostras em outro sistema de eixo mais conveniente para a análise dos dados. Em outras palavras, as n-variáveis originais geram, através de suas combinações lineares, n-componentes principais, cuja principal característica, além da ortogonalidade, é que são obtidos em ordem decrescente de máxima variância, ou seja, a componente principal 1 detém mais informação estatística que a componente principal 2, que por sua vez tem mais informação estatística que a componente principal 3 e assim por diante. Este método permite a redução da dimensionalidade dos pontos representativos das amostras’, pois, embora a informação estatística presente nas n-variáveis originais seja a mesma dos n-componentes principais, é comum obter em apenas 2 ou 3 das primeiras componentes principais mais que 90% desta informação (MILLER; MILLER, 2005).

O gráfico da componente principal 1 versus a componente principal 2 fornece uma janela privilegiada (estatisticamente) para observação dos pontos no espaço n-dimensional. A análise de componentes principais também pode ser usada para julgar a importância das próprias variáveis originais escolhidas, ou seja, as variáveis originais com maior peso “loadings” na combinação linear dos primeiros componentes principais são as mais importantes do ponto de vista estatístico. Portanto, a tarefa do químico que trabalha com estatística multivariada, consiste em interpretar a distribuição dos pontos no gráfico de componentes principais e identificar as variáveis originais com maior peso na combinação linear das componentes principais mais importantes (MOITA NETO; MOITA, 1998).

2.2.9.3 Modelagem independente para analogia de classes

O “Soft Independent Modeling of Class Analogy” (SIMCA) é um método quimiométrico probabilistico e modelativo fundamentado na análise por componentes principais, onde para cada classe de amostra, cria-se um modelo PCA separadamente, onde são construidos envelopes em torno de cada classe de amostra de acordo com as componentes principais que melhor agrupan as classes (BRERETON, 2007).

2.2.9.4 Método baseado no vizinho mais proximo

O “K-Nearest Neighbord” (KNN) é utilizada a comparação entre as distâncias das amostras, calculando-se para cada par de amostras a distância Euclidiana, com base na proximidade às amostras do conjunto de treinamento. Para validação dos dados utiliza-se validação cruzada, onde cada amostra é retirada e testada todas as outras, definindo sua classe. A classe é determinada a partir de quantas amostras, da mesma classe, estão mais perto da amostra selecionada. Aquela classe que tiver maior numero de vizinhos a amostra, será a classe atribida à amostras (MILLER; MILLER, 2005)

Existem pacotes computacionais de estatística que fazem todas as operações necessárias à obtenção de componentes principais e agrupamento hierárquico, inclusive o tratamento prévio de padronização e escalonamento dos dados, como é o caso do SPSS, SYSTAT, PIROUETTE, etc. No “Statistical Package for the Social Sciences” (SPSS), a opção de componentes principais aparece no menu através de uma de suas finalidades: a redução de dados. As componentes principais também podem ser obtidas como um dos métodos da análise de fatores (Factor Analysis) (MOITA NETO; MOITA, 1998)

2.3 CROMATOGRAFIA

2.3.1 Conceito e histórico

Atribuída ao botânico russo Mikhael Semenovich Tswett, a descoberta da cromatografia como técnica analítica ocorreu em 1906, quando descreveu sua experiência na separação dos componentes de extrato de folhas. Neste estudo, o botânico conseguiu separar pigmentos de cloroplastos em folhas verdes de plantas, onde usou uma coluna de vidro recheada com carbonato de cálcio como fase estacionária e éter de petróleo como fase móvel, ocorrendo a separação de componentes em faixas coloridas, este fato deu origem ao nome de cromatografia (*chrom* = cor e *grafie* = escrita) embora o processo não dependa da cor (COLLINS, 1997).

A técnica cromatográfica foi praticamente ignorada até a década de 30 quando foi redescoberta por Kuhn e Lederer que aperfeiçoaram a cromatografia em coluna, separando e identificando as xantofilas da gema de ovo, utilizando um experimento semelhante ao de Tswett, com carbonato de cálcio como fase estacionária e éter de

petróleo como fase móvel. A partir daí a cromatografia foi aperfeiçoada e em conjunto com os avanços tecnológicos foi levada a um alto grau de sofisticação que resultou no seu grande potencial de aplicação em muitas áreas (COLLINS, 1997).

2.3.2 Cromatografia Liquida de Alta Eficiência

A cromatografia pode ser utilizada para a identificação de compostos, por comparação com padrões previamente existentes para a purificação de compostos separando-se as substâncias indesejáveis e para a separação dos componentes de uma mistura.

2.3.2.1 Instrumentação

Existe uma série de componentes que podem ser utilizados para conformar um sistema de CLAE os quais podem variar conforme a determinação de substâncias que se tem projetado. Diversos autores (SIOUFFI, 2000; LANFRANCO et al., 2010; NICOLE; PAUL, 2010) têm mostrado os componentes básicos para um sistema os quais são descritos a seguir:

Os reservatórios de fases móveis utilizadas no CLAE podem ser de vidro ou aço inoxidável, o volume pode variar conforme a frequência de uso. Os reservatórios frequentemente estão equipados com um filtro para remover partículas que possam obstruir e danificar o sistema de bombeamento e a coluna e um sistema de remoção dos gases dissolvidos usualmente oxigênio e nitrogênio que interferem formando bolhas na coluna e nos sistemas de detecção.

Os requisitos para um sistema de bombeamento da CLAE são rigorosos e incluem: (1) geração de pressões até 6000 psi (libra/polegada2), (2) saída com ausência de pulsos, (3) velocidades de pulso variando de 0,1 a 10 mL/min, (4) controle de fluxo e reprodutibilidade relativa de fluxo de 0,5% ou ainda melhor, e (5) componentes resistentes à corrosão, vedação de aço inoxidável ou Teflon. As bombas mais comuns são aquelas que possuem um êmbolo ou pistão que é deslocado de forma contínua e uniforme por um motor de precisão, comprimindo o líquido contido em uma câmara de volume constante, contudo na atualidade existem bombas binárias, ternárias e quaternárias que possibilitam uma melhor separação e eficiência do CLAE.

As válvulas de injeção usadas possuem uma alça de amostragem (em inglês “loop”) para a introdução da amostra com uma seringa e duas posições, uma para o preenchimento da alça e outra para a sua liberação para a coluna. Existem alças de diversos volumes, sendo utilizadas geralmente, alças na faixa de 5-50µL para injeções analíticas e 0,5-2 mL para preparativas.

As colunas utilizadas em CLAE são geralmente de aço inoxidável, com diâmetro interno entorno de 0,45 cm para separações analíticas e na faixa de 2,2 cm para preparativas. Os comprimentos são variáveis sendo comuns colunas analíticas de 10-25 cm e preparativas em torno de 25-30 cm. Essas colunas são reaproveitáveis, sendo empacotadas com suportes de alta resolução, não sendo necessária sua regeneração após cada separação.

Os detectores utilizados para separações no CLAE são de dois tipos básicos, os de propriedades universais ou globais que respondem a propriedades da fase móvel como um todo, como índice de refração, constante dielétrica ou densidade, que é modulada pela presença de solutos. Ao contrário, detectores de propriedades do soluto respondem a algumas propriedades do soluto, como absorbância no UV, fluorescência ou corrente de difusão que não pertencem à fase móvel. O registro pode ser efetuado usando registrador, integrador ou microcomputador.

Espectrofotômetros de comprimentos de onda variável UV-VIS, cobrindo a faixa de 190-800 nm, através de monocromador que seleciona o comprimento de onda desejado do feixe de luz emitido pelas lâmpadas de deutério (UV) ou tungstênio (VIS). Oferecem várias vantagens sobre os instrumentos de comprimento de onda fixo como uma alta absorbância para vários componentes devido à escolha de comprimento de onda e permite maior seletividade, desde que um determinado comprimento de onda pode ser escolhido. Análises simultâneas, em vários comprimentos de onda também são possíveis por médio de um arranjo de diodos que consiste em uma série de fotodiodos detectores posicionados lado a lado num cristal de silício de modo que cada comprimento de onda difratado pela grade atinge um ponto deste arranjo, e consequentemente um detector.

2.3.2.2 Etapas aplicadas para a determinação de aminas utilizando o CLAE

De uma perspectiva analítica para determinação de aminas em matrizes alimentares utilizando a cromatografia liquida de alta eficiência a maioria das

técnicas envolvem as etapas de extração, derivatização, separação e quantificação (ÖNAL, 2007; LANFRANCO et al., 2010).

O tipo e a natureza das aminas e dos alimentos a serem analisados afetam significativamente a eficiência da extração. A extração de aminas de uma matriz sólida pode ser realizada utilizando-se solventes orgânicos, como metanol, acetona ou etanol ou reagentes ácidos, como ácido clorídrico, ácido perclórico e ácido tricloroacético os quais são adicionados na matriz alimentar previamente homogeneizada (ÖNAL, 2007; LANFRANCO et al., 2010).

A detecção de aminas em matrizes alimentares complexas torna-se difícil devido ao fato de estarem presentes em baixas concentrações. Além disto, a maioria das aminas não apresenta absorção no Ultra-Violeta (UV) e nem fluorescência, tornando-se necessário um processo de derivatização para aumentar a absorbância e, consequentemente, diminuir o limite de detecção. Alguns dos reagentes de derivatização normalmente empregados na análise de aminas são 9-fluorenilmetil cloroformato, naftaleno-2,3-dicarboxialdeído, cloreto de 5-dimetilaminonaftaleno sulfonila (cloreto de dansila), fluorescamina, e o-ftalaldeído (OPA) (ÖNAL, 2007; LANFRANCO et al., 2010).

A separação e quantificação das aminas biogênicas por CLAE oferecem maiores vantagens sobre outros métodos, pois este permite a separação e quantificação simultânea. A CLAE por fase reversa (fase estacionária apolar e uma fase móvel moderadamente polar) é considerada a técnica mais adequada para análise de aminas em alimentos. Este método opera mediante o principio da interação hidrofóbica o qual resulta das forças repulsivas entre o eluente polar e a fase estacionaria apolar (BANSAL et al., 2010).

2.3.3 Validação de métodos cromatográficos

Vários autores definem validação de métodos e pode-se dizer que os conceitos continuam evoluindo e estão constantemente sob consideração pelas agências reguladoras. Algumas definições descritas na revisão feita por RIBANI et al. (2004) são:

“A validação deve garantir, através de estudos experimentais, que o método atenda às exigências das aplicações analíticas, assegurando a confiabilidade dos resultados” (BRASIL, 2003).

“Validação é o processo de definir uma exigência analítica e confirmar que o método sob investigação tem capacidade de desempenho consistente com o que a aplicação requer” (EURACHEM, 1998).

“Confirmação por testes e apresentação de evidências objetivas de que determinados requisitos são preenchidos para um dado uso intencional” (ISO, 1999).

2.3.3.1 Seletividade

A seletividade de um método instrumental de separação é a capacidade de avaliar, de forma inequívoca, as substâncias em exame na presença de componentes que podem interferir com a sua determinação em uma amostra complexa. Na seletividade é avaliado o grau de interferência de espécies como outro ingrediente ativo, excipientes, impurezas e produtos de degradação, bem como outros compostos de propriedades similares que possam estar, porventura, presentes. A seletividade garante que o pico de resposta seja exclusivamente do composto de interesse (Figura 4) (RIBANI et al., 2004).

2.3.3.2 Linearidade

A linearidade corresponde à capacidade do método em fornecer resultados diretamente proporcionais à concentração da substância em exame. A correlação entre o sinal medido (área ou altura do pico) e a massa ou concentração da espécie pode ser expressa mediante a equação da reta ($x = ay + b$) chamada *curva analítica* (Figura 5). Além dos coeficientes de regressão a e b, também é possível calcular, a partir dos pontos experimentais, o coeficiente de correlação **r**. Este parâmetro permite uma estimativa da qualidade da curva obtida, pois quanto mais próximo de 1,0, menor a dispersão do conjunto de pontos experimentais e menor a incerteza dos coeficientes de regressão estimados. Um coeficiente de correlação maior que 0,999 é considerado como evidência de um ajuste ideal dos dados para a linha de regressão (RIBANI et al., 2004)

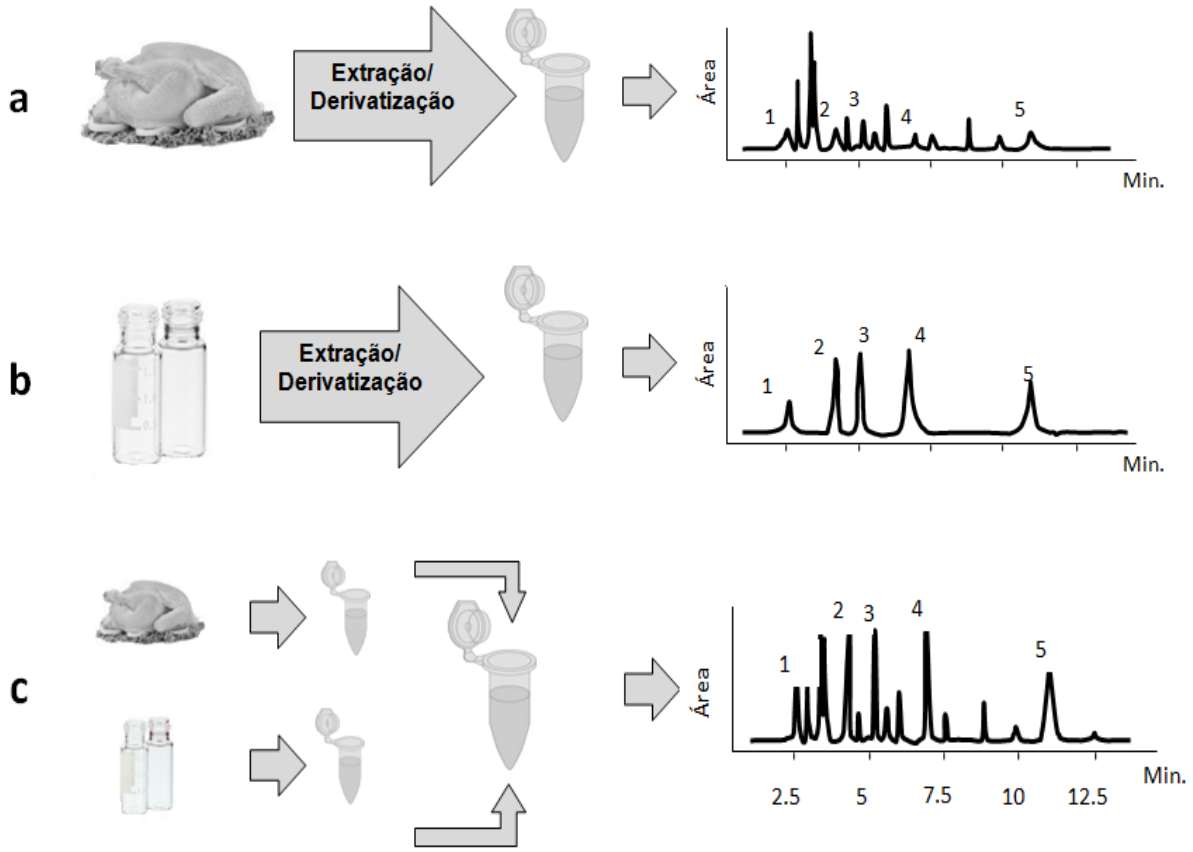


Figura 4. Desenho da seletividade. a) Extração e derivatização da amostra (carne de frango) e cromatograma; b) Extração e derivatização dos padrões (aminas biogenicas) e cromatograma; e c) Amostra adicionada dos padrões e cromatograma.

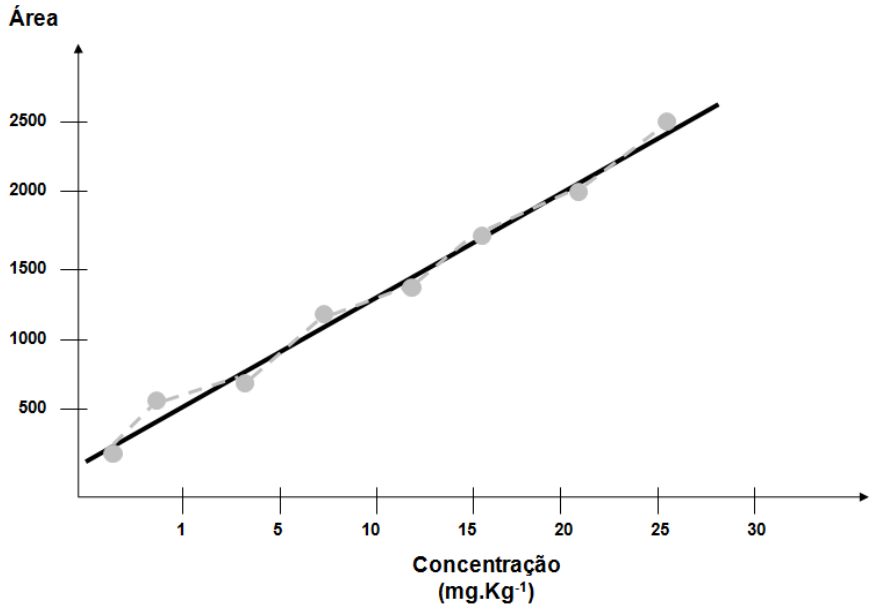


Figura 5. Curva analítica clássica da linearidade.

2.3.3.3 Precisão

A precisão de um método bioanalítico é a medida dos erros aleatórios e representa a proximidade dos resultados obtidos a partir de medidas independentes de amostragens múltiplas de uma amostra homogênea. Este é um importante parâmetro que possibilita decidir se o método bioanalítico é confiável ou não para o objetivo da análise. A precisão pode ser expressa como uma estimativa do desvio padrão ou Desvio Padrão Relativo (DPR), também conhecido como Coeficiente de Variação (CV%), de uma série de repetições da mesma amostra, em diferentes preparações (CASSIANO et al., 2009).

A precisão em validação de métodos é considerada em três níveis diferentes: repetitividade; precisão intermediária; reprodutibilidade. Na repetitividade é definida a precisão do método em repetir, em um curto intervalo de tempo, os resultados obtidos nas mesmas condições de análise, contudo, com o mesmo analista, com o mesmo equipamento, no mesmo laboratório e fazendo uso dos mesmos reagentes (CASSIANO et al., 2009).

A precisão intermediária é a habilidade do método em fornecer os mesmos resultados quando as análises são conduzidas no mesmo laboratório, mas em diferentes dias, por diferentes analistas e diferentes equipamentos. Para a determinação da precisão intermediária, recomenda-se um mínimo de dois dias diferentes com analistas diferentes e, na maioria das validações bioanalíticas, adotam-se três dias não consecutivos (CASSIANO et al., 2009).

A reprodutividade é o termo utilizado para demonstrar a precisão entre laboratórios. Os resultados são obtidos usando o mesmo método e mesmas amostras em diferentes laboratórios, diferentes analistas e equipamentos (CASSIANO et al., 2009).

2.4.2.4 Recuperação

A recuperação do analito pode ser estimada pela análise de amostras adicionadas com quantidades conhecidas do mesmo analito.A percentagem de recuperação pode ser estabelecida pela seguinte formula:

$$\textbf{Recuperação (\%)} = \frac{\textbf{C1 – C2}}{\textbf{C3}}$$

onde:

C1 = concentração determinada na amostra adicionada.

C2 = concentração determinada na amostra não adicionada.

C3 = concentração adicionada.

2.3.3.5 Limite de Detecção

O limite de detecção (LOD, do inglês “Limit Of Detection”) representa a menor concentração da substância em exame que pode ser detectada, mas não necessariamente quantificada, utilizando um determinado procedimento experimental (RIBANI et al., 2004).

O LD pode ser calculado pelo método visual utilizando a matriz alimentar com adição de concentrações conhecidas da substância de interesse, de tal modo que se possa distinguir entre ruído e sinal analítico pela visualização da menor concentração visível (detectável) (RIBANI et al., 2004). Também pode ser calculado pelo método da relação sinal-ruído que é feita a comparação entre a medição dos sinais de amostras em baixas concentrações conhecidas do composto de interesse na matriz e um branco (matriz isenta do composto de interesse) destas amostras. Assim, é estabelecida uma concentração mínima na qual a substância pode ser facilmente detectada. A relação sinal-ruído pode ser de 3:1 ou 2:1, proporções geralmente aceitas como estimativas do limite de detecção.

2.3.3.6 Limite de Quantificação

O limite de quantificação (LOQ, do inglês “Limit Of Quantitation”) representa a menor concentração da substância em exame que pode ser mesurada, utilizando um determinado procedimento experimental. Como o LD, o LQ é expresso como uma concentração, sendo que a precisão e a exatidão das determinações também devem ser registradas. Os mesmos critérios de LOD podem ser adotados para o LOQ, utilizando a relação 10:1, ou seja, o LQ pode ser calculado utilizando o método visual ou a relação sinal-ruído (RIBANI et al., 2004).

2.3.3.7 Robustez

Segundo EURACHEM, um método é robusto quando não é afetado por uma modificação pequena e deliberada em seus parâmetros. A robustez de um método cromatográfico é avaliada, por exemplo, pela variação de parâmetros como a concentração do solvente orgânico, pH e força iônica da fase móvel em HPLC,

programação da temperatura, o tempo de extração, agitação, etc. As mudanças introduzidas refletem as alterações que podem ocorrer quando um método é transferido para outros laboratórios, analistas ou equipamentos EURACHEM (1998).

2.5 RADIAÇÃO ULTRAVIOLETA

A luz ultravioleta (UV) tem o histórico de ter sido utilizada como tratamento antibacteriano e na atualidade esta ganhando aceitação na indústria alimentar como um efetivo bactericida. A indústria avícola tem se interessado por esta tecnologia devido a que é um processo não térmico que pode ser efetivo na eliminação de patógenos (WALLNER-PENDLETON et al., 1994).

A radiação ultravioleta foi descoberta em 1801 pelo cientista alemão Johan Ritter, que percebeu uma forma invisível de luz além do violeta capaz de oxidar haletos de prata, chamada de luz ultravioleta no fim do século XIX. Ocupa ampla faixa de comprimento de onda na região não ionizante do espectro eletromagnético, entre os raios X (100 nm) e a luz visível (400 nm) cujas subdivisões podem se observar no Quadro 3.

Quadro 3. Características da luz ultravioleta

Tipo	Comprimento de onda	Faixa (nm)	Características
UV-A	Longo	320-400	Alterações na pele humana (bronzeamento)
UV-B	Médio	280-320	Queimadura da pele (câncer)
UV-C	Curto	200-280	Faixa germicida (microrganismos)
UV-V		100-200	Região de UV de vácuo

Fonte: Guerrero-Beltrán; Barbosa-Cánovas (2004)

A tecnologia de irradiação por UV é aplicada, desde 1930 nos Estados Unidos, em superfícies e no ar, em ambientes estéreis como hospitais. Depois foi adaptada para a esterilização de embalagens no sistema Ultra High Temperature (UHT), tais como tampas de garrafas de polietileno de alta densidade e cartões para produtos líquidos, embalagens de iogurte, copos plásticos e tampas de alumínio, além de

superfícies de frutas e hortaliças para aumentar a resistência dos tecidos a microrganismos deterioradores (BINTSIS et al., 2000).

A fonte natural mais importante de UV é o sol podendo chegar na superfície da terra, mas a intensidade da faixa UV-C é retida nas atmosferas superior e sua passagem depende da quantidade de ozônio e oxigeno. Artificialmente, existe uma serie de lâmpadas de vapor de mercúrio de baixa pressão. São cobertas por material que permite a transmissão de radiação UV-C adequada para produzir energia na região germicida (aproximadamente 254 nm). As lâmpadas de quartzo possuem maior transmitância, no entanto, seu elevado custo faz com que sejam substituídas pelas de vidro (com níveis aceitáveis de transmitância). A radiação atravessa o tubo de quartzo ou vidro e atinge os microrganismos que estão localizados no ar ou no líquido em volta da lâmpada (GUEDES et al., 2009).

2.5.1 Efeitos nos microrganismos

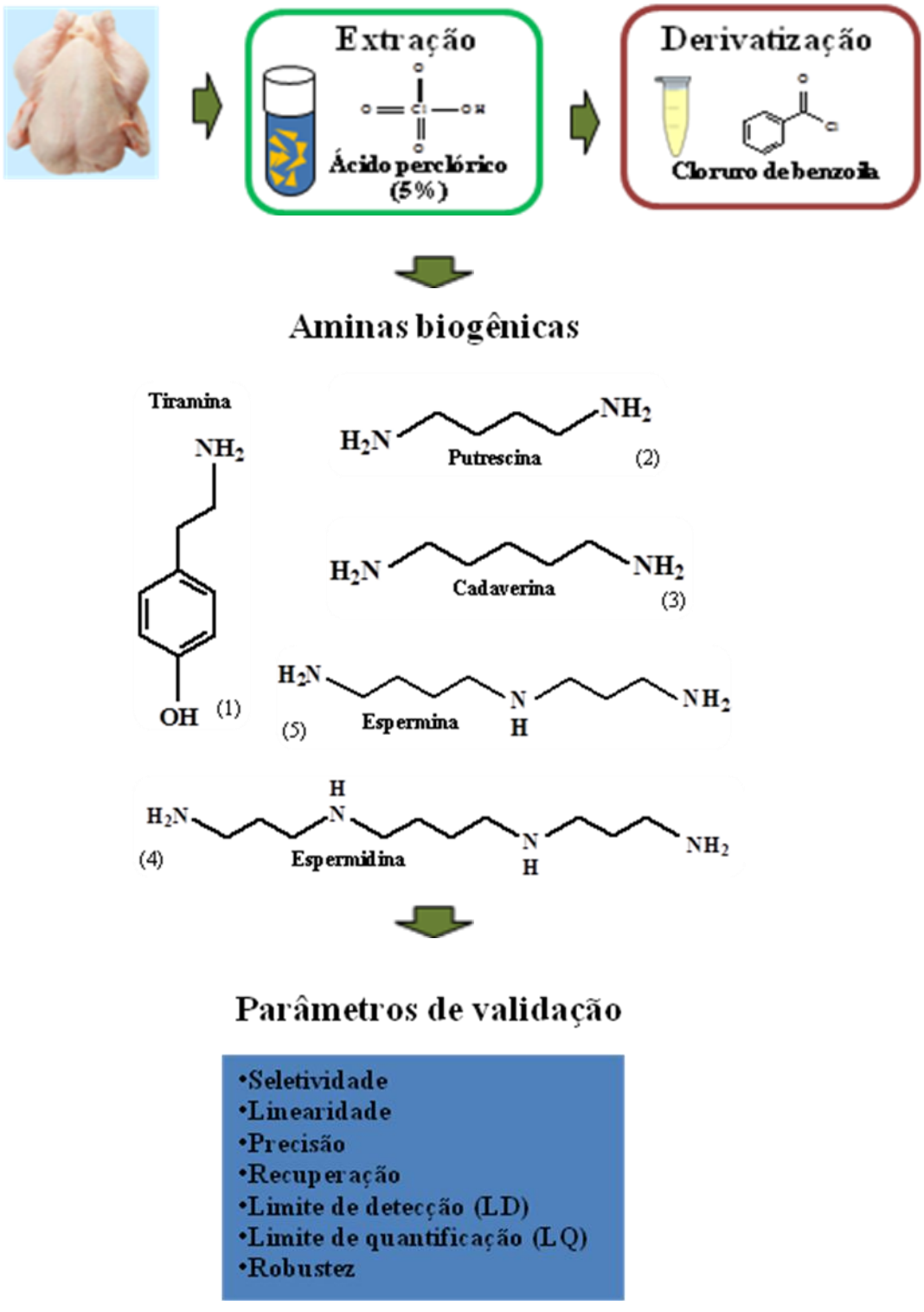
O efeito da luz UV pode variar entre espécies e na mesma espécie pode ser afetado pela cepa, meio de cultura, fase de crescimento, densidade e outras características vinculadas com a composição do alimento. O principal efeito da luz UV é ao nível dos ácidos nucleicos produzindo um deslocamento físico dos elétrons o que ocasiona a divisão dos enlaces de DNA o que pode deter o crescimento e induzir uma morte celular.

2.5.2 Regulações internacionais

Diversos países tem adotado o uso de esta tecnologia para a conservação dos alimentos. A Administração de alimentos e bebidas dos Estados Unidos (FDA, do inglês “Food and Drug Administration”) aprovou no ano 2000 o uso da luz UV como alternativa para o tratamento termal da pasteurização em sucos na eliminação de patógenos e outros microorganismos. O departamento de “Novel foods” no Canadá também foi aprovada a utilização de uma unidade UV para cidra e suco de maça, indicando que a aplicação de esta tecnologia não tem risco para a saúde dos consumidores. Finalmente a União Europeia considera à luz UV como irradiação, e na Europa o uso de irradiação em alimentos não esta regulada (KOUTCHMA et al., 2009).

3 DESENVOLVIMENTO

3.1 ARTIGO 1: VALIDATION OF AN HPLC METHODOLOGY FOR THE IDENTIFICATION AND QUANTIFICATION OF BIOGENIC AMINES IN CHICKEN MEAT. Published in Food Analytical Methods (Paper I)



VALIDATION OF AN HPLC METHODOLOGY FOR THE IDENTIFICATION AND QUANTIFICATION OF BIOGENIC AMINES IN CHICKEN MEAT

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Abstract

This study validated a high performance liquid chromatography (HPLC) method to determine biogenic amines in chicken meat. For the identification of amines, an isocratic elution system coupled with a UV detector (254 nm) was used, after a perchloric acid (5%) extraction and benzoyl chloride derivatization of the samples. The standards of tyramine, putrescine, cadaverine, spermidine and spermine were used for the following validation parameters: selectivity, linearity, accuracy, recovery, limit of detection and quantification, and robustness. Finally, chicken breasts commercialized in two types of packaging were evaluated. The results showed a excellent selectivity and separation of all amines, $r^2 > 0.99$, RDS < 5%, recovery between 64% and 112%, limits of detection and quantification between 0.03 - 1.25 µg L^{-1} and 0.15 - 5.00 µg L^{-1}, respectively, and appropriate robustness for the proposed methodology. Moreover, both chicken meat commercial packages had similar values for all amines; only tyramine was significantly different ($p \leq 0.05$). The proposed method was suitable to detection and quantification of simultaneous five biogenic amines in chicken meat.

Keywords *validation, HPLC, biogenic amines, meat, chicken.*

Introduction

The Brazilian production of chicken meat in 2010 reached 12,230 million tons, a growth of 11.38% in comparison with 2009; this increase was mainly due to internal consumption and exportation. With this performance, Brazil approaches China, currently the second-largest chicken producer in the world at 12,550 million tons, below only the United States at 16,648 million tons, according to projections of the Department of Agriculture of the United States [1].

Due to increases in the global demand for chicken meat, poultry suppliers are obliged to implement specific controls to guarantee food safety and a high quality product. The meat is especially susceptible to protein degradation, and the determination of substances that originate by this process can be used as quality indicators in meat [2]. Biogenic amines are low molecular weight substances, primarily produced by amino acid decarboxylase enzymes produced by some microorganisms. The presence of these molecules in foods is directly related to amino acid composition, microflora, storage temperature, maturation time, packing, and other factors [3].

Reliable methods for evaluating biogenic amine production are important for preventing food-borne intoxication, maintaining good control of the production chain and checking the safety quality. High performance liquid chromatography methods are reliable and highly sensitive techniques for the simultaneous detection and quantification of different biogenic amines [4].

Validation is a process that is used to authenticate that the analytical procedure employed for a specific test is suitable for its intended use [5]. In addition, it confirms good performance, reduces analytical errors, and improves the quality, reliability and reproducibility of the method under consideration [6]. New analytical methods need to be validated in an objective way to demonstrate their application for pre-determined usefulness [7]. Non-validated methods can generate unreliable results and are not official recognized by national and international authorities [8].

Trustworthy results are a prerequisite for the interpretation and correct evaluation of scientific research and routine laboratory analyses. Unreliable results will over- or underestimate the possible effects of analyzed metabolites and lead to false interpretations and unjustified conclusions. For this reason, the aim of this study was to validate a methodology for biogenic amine determination in chicken meat using high performance liquid chromatography.

Materials and Methods

Standards preparation

Standards of tyramine ($C_8H_{11}NO$), putrescine ($C_4H_{12}N_2$), cadaverine ($C_5H_{14}N_2$), spermidine ($C_{14}H_{47}N_6O_{12}P_3$) and spermine ($C_{10}H_{26}N_4$) were purchased from Sigma-Aldrich® (St. Louis, Missouri, USA). Stock solutions for each amine (40 μg L^{-1}) were prepared in 0.1N HCl and stored at 4±1°C. Different dilutions were prepared from the stock solutions to predetermined concentrations for each validation phase. A 2*N* solution of NaOH was added to all diluted stock amine solutions until the pH was greater than 12. Samples were derivatized using benzoyl chloride (40 μL); samples were homogenized by vortexing for 15 seconds and were then kept at room temperature for 20 minutes. The mixture was extracted two times with 1000 μL of diethyl ether. The ether layer was aspirated and evaporated to dryness under a stream of nitrogen (Sample Concentrator Techne®, Cambridge, UK). Finally, the residue was dissolved in 1000 μL of the mobile phase and stored at 4±1°C.

Sample preparation

Two commercial packages (an expanded polystyrene tray and a low-density polyethylene bag) of frozen chicken breasts (*Gallus gallus domesticus*) were purchased (n = 10 for each package) at commercial markets in Rio de Janeiro, Brazil, and transported to the laboratory in insulated polystyrene boxes on ice. For amine extraction, 5 g of minced breast chicken meat was homogenized with 5 mL of 5% perchloric acid. The homogenates were kept under refrigeration (4±2ºC) for 1 hour and shaken continuously (Certomat® MV, B. Braun Biotech International, Melsungen, Germany); then, the mixture was centrifuged at 503 *g* for 10 minutes at 4±1ºC (Hermle Z 360 K) and filtered through Whatman No. 1 filter paper. The filtrates were neutralized (pH > 6) with 2*N* NaOH and kept in an ice bath (0±2ºC) for approximately 20 minutes, followed by a second filtration and addition of NaOH (pH > 12) under the same conditions. The derivatization procedure was carried out in the same way as for the standards.

Chromatographic conditions

The chromatographic system consisted of a LC/10AS pump (Shimadzu, Kyoto, Japan) coupled to a SPD/10AV UV-Vis detector (Shimadzu) and a C-R6A chromatopack integrator (Shimadzu). Amine separations were performed on a Teknokroma Tracer Extrasil ODS2 (15 x 0.46 cm id., 5 μm) column equipped with a Supelco Ascentis C18 (2 x 0.40 cm, id. 5μm) guard column, under isocratic conditions. The mobile phase was prepared by mixing

acetonitrile (Tedia®) and Milli-Q water (Simplicity UV, Millipore, Molsheim, France), 42:58 (v/v); the mixture was degassed in an ultrasonic bath (Cleaner USC 2800 A). The flow rate was 1 $mL.min^{-1}$, the injected volume was 20 µL, the column temperature was 20°C and the detector wavelength was set at 198 nm. Injections were performed using a 50 µL syringe (Hamilton microliter TM 705) and the total run time was 15 minutes. An injection of pure acetonitrile for 10 minutes was used between each sample to flush the HPLC system. The biogenic amines were identified by retention time and were quantified by peak area.

Validation parameters

The method for the identification of biogenic amines in chicken meat was validated in terms of the analytical parameters of selectivity, linearity, precision, recovery, limit of quantification, limit of detection and robustness following conventional protocols from international guidelines [9, 10].

Selectivity was performed injected different concentrations (50, 75, 100, and 125 mg L^{-1}) of each biogenic amine standard and compared with the chromatogram of the chicken meat spiked with the same biogenic amine standard concentration. Likewise, a mixed solution of five biogenic amines, a chicken meat sample and a chicken meat sample spiked with the mix solution were injected and evaluated the retention times and the separation of each amine.

Linearity was determinate injected eight sample concentrations (1, 10, 12.5, 25, 50, 100, 125, and 250 mg L^{-1}) of each amine standard in three times onto the HPLC. Linear calibration curves were constructed and the regression equation and the regression coefficient (r^2) were calculated for each biogenic amine.

Accuracy was considered at two levels: (1) repeatability, it was established with three different concentrations of amine mix standard: solution A (100 mg L^{-1} of putrescine, cadaverine, spermidine, spermine, and 300 mg L^{-1} of tyramine); solution B (200 mg L^{-1} of putrescine, cadaverine, spermidine, spermine, and 500 mg L^{-1} of tyramine); and solution C (250 mg L^{-1} of putrescine, cadaverine, spermidine, spermine, and 1000 mg L^{-1} of tyramine), injected ten times and expressed as the mean, standard deviation (SD) and relative standard deviation (RSD); and (2) intermediate precision, performed with a mix solution (200 mg L^{-1} of putrescine, cadaverine, spermidine, spermine and 400 mg L^{-1} of tyramine) injected five times. This procedure was repeated on three consecutive days and expressed in the same way for repeatability.

Recovery was tested by the standard addition procedure injected three amine concentrations (100, 150 and 200 mg L^{-1}). The following equation, R = [(C-A)/B] x 100, was used with chicken meat samples (A), standard amines (B), and meat chicken samples fortified with standard amines (C), prepared and injected in triplicate.

The determination of LOD was based on visual evaluation. For this purpose, smaller concentrations of each amine calibration curve were injected in a decreasing sequence until the chromatographic signal reached an area that could be visually differentiated from the signal noise (baseline) at the lowest attenuation. When this area was identified, three injections of each solution were performed for confirmation. LOQ was calculated in the same way as LOD.

The evaluation of robustness was planned with small variations in four parameters: mobile phase of acetonitrile:water (42:58, 43:57 and 41:59); wavelength (197, 198 and 199 nm); flow rate (0.95; 1.00 and 1.05 $mL.min^{-1}$), and derivatization time (15, 20 and 25 minutes). For all parameters a standard solution mixture of putrescine, cadaverine, spermidine and spermine (200 mg L^{-1}) and tyramine (400 mg L^{-1}) was used.

Statistical analysis

Data collected in this study were analysed using GraphPad Prism® 5.00 package [11] for windows by one-way analysis of variance (ANOVA) and the means were compared with Tukey test ($p < 0.05$).

Results and Discussion

Calibration curves of biogenic amine standards and chicken meat samples spiked with the amine standards were compared (Fig. 1). The curves were parallel, suggesting that there was no matrix interference. Food samples may contain components that interfere with performance measurement and may increase or decrease the signal detector [5]. Additionally, the chromatograms for each amine standard, amine mix solution, chicken meat and spiked chicken meat showed no interference at their respective retention times (tyramine: 2.32, putrescine: 4.35, cadaverine: 5.25, spermidine: 7.12 and spermine: 11.70). The separation of amines from other components in the matrix was effective (Fig. 2). This fact can be explained by the separation ability of the ODS2 column (15 x 0.46 cm id. 5 μm). The same stationary phase was used by Baston et al. [8] reported a good separation of amine standards in chicken meat samples. Errors of determination in samples can be considered a problem of analyte

detection or low efficiency in the extraction. This result confirms the selectivity of the proposed method [6].

The regression equation and regression coefficient (r^2) can be observed in the Table 1. The r^2 values were between 0.9997 and 0.9921 which are compatible with an optimal setting. These results were possible due to the use of eight different concentrations for the construction of the calibration curve, which showed a best-fit linear regression model. The European Community recommends at least five concentration levels for the construction of calibration curves [12]. The results are consistent with Brazilian legislation and INMETRO, which consider 0.99 and 0.90 a excellent r^2 value, respectively [13, 14]. Other researchers show an optimal data adjustment for the regression coefficient ($r^2 = 0.99$) for the same amines (tyramine, putrescine, cadaverine, spermine and spermidine) [8, 15]. The method developed was linear for the concentration range 1-250 mg L^{-1}, with r^2 values above 0.99 for all of the amines studied.

The RSD values were less than 2.5% in the three concentrations of amine mix standards used for repeatability (Table 2). In assessing the accuracy, previous literature had determined that an RSD equal to or less than 5% was an acceptable value for bioanalytical methods [14] and up to 20% in methods for quantifying trace elements [16]. These results were similar to previous research, with values for RSD less than 3.87% for the identification of biogenic amines in chicken meat [15].

For intermediate precision, the RSD results showed no significant difference ($P > 0.05$) for tyramine, putrescine, spermidine and spermine on three different days (Table 3). However, cadaverine values were different on the first day ($P \leq 0.05$). Accuracy results based on the RSD, obtained on different days, were considered acceptable, being within the limit for validation of chromatographic methods [14-16]. No significant differences ($P > 0.05$) were observed in retention times for the three days in all amine standards.

The recovery was better in solutions prepared with high amine standard concentrations (Table 4). These results differ from Baston et al. [8], they used three different concentrations (0.5, 1.0 and 2.0 mg L^{-1}) and described a excellent recovery for spermine (> 99%) and cadaverine and spermidine (> 95%); and relatively low for putrescine (> 94%) and tyramine (> 92%) at all concentrations. These authors also indicated that recoveries exceeding 100% are normal but it should not exceed 105%, which indicates an equipment problem (column and detector). The variations in recovery may be related to changes in the methodology, filters with smaller diameter pores, derivatization substances, different concentrations, gradient systems, and

other factors. Vinci and Antonelli [17] reported that parameters such as solvent, extraction, pH and conditions chosen for derivatization could influence recovery values, but in this case, the same methodology was used for all determinations.

LOD and LOQ values were determined by the injection of serial dilutions from the lowest amine concentration used in the linearity. Tyramine and cadaverine presented the lowest and highest values, respectively, for LOD and LOQ (Table 4). Baston et al. [8] found values between 5 to 30 µg L^{-1} for LOD and 10 to 60 µg L^{-1} for LOQ for the same amines; however, their protocol used a signal-to-noise ratio and wavelength of 254 nm for the determination of limits. The most important factors that determined these results were the higher sensitivity of the detector wavelength (198 nm) used in the HPLC system and the derivatized substance (benzoyl chloride), which in addition to the advantage of rapid derivatization, has a better stability than other derivatizing agents, such as o-phthalaldehyde or dansyl [18].

The results of robustness in different parameters are showed in table 5. Changes in mobile phase concentration did not modify the amine determination. The values for putrescine are significantly different ($P \leq 0.05$) using the mobile phase concentration of 41:59. For wavelength variations, cadaverine and spermine showed significant differences ($P \leq 0.05$) at 197 nm. Baston et al. [8] found little difference in the results using two wavelength variations (249 and 259 nm).

Another parameter evaluated was flow rate. This variable was associated with significant differences ($P \leq 0.05$) for putrescine and tyramine at 1.05 and 0.95 $mL.min^{-1}$, respectively. For derivatization time, 15 minutes was significantly different ($p \leq 0.05$) for all standard amines, indicating that 15 minutes is insufficient and that 20 minutes or more is required for appropriate derivatization. In general, the robustness results were satisfactory for the proposed method.

Tyramine had the highest value in chicken breast meat from the two types of commercial packaging (Table 6). Both presentations were significantly different ($P \leq 0.05$) for tyramine; no differences ($P > 0.05$) were observed for the other amines. These results are consistent with those described by Rokka et al. [19], who found 100 $mg.Kg^{-1}$ of tyramine, 82 $mg.Kg^{-1}$ for spermine and less than 15 $mg.Kg^{-1}$ for putrescine, cadaverine and spermidine in chicken meat stored under refrigeration for 12 days.

Vinci and Antonelli [17] compared amine levels produced by beef and chicken meat and observed that chicken meat conservation was critical because non-physiological biogenic

amines increased earlier and more rapidly than in beef. They attribute this result to differences in chicken muscles, where there are shorter fibers that can be easily attacked by proteolytic enzymes, increasing the amount of amino acid precursors responsible for amine biosynthesis.

Muscle cells rupture and, consequently, amino acid release can be attributed to freezing temperatures. Ruiz-Capillas and Jiménez-Colmenero [20] proposed that the freezing process can lead to structural and chemical changes in meat and that these modifications depend on chilling treatments and storage conditions. Kozová et al. [21] found values of 30-70 mg L^{-1} for spermidine and 250-300 mg L^{-1} for spermine in chicken meat frozen for six months. According to Silva and Gloria [22], spermine values can be reduced during storage time under refrigeration, where some microorganisms use this polyamine as a source of nitrogen.

Other researchers present results with significant variations. Sander et al. [23] found high levels of cadaverine (541.19 mg.Kg^{-1}), putrescine (244.19 mg.Kg^{-1}) and tyramine (220.39 mg.Kg^{-1}) and low of spermine (4.08 mg.Kg^{-1}) and spermidine (5.72 mg.Kg^{-1}) in fresh chicken carcasses. Balamatsia et al. [24] described levels of tyramine and cadaverine at less than 10 mg.Kg^{-1} and initial values of putrescine exceeding 48 mg.Kg^{-1} and spermine at 53 mg.Kg^{-1}. Gallas et al. [25] reported concentrations of spermine (17.9 mg L^{-1}), putrescine (26.4 mg L^{-1}), cadaverine (8.5 mg L^{-1}) and spermidine (7.3 mg L^{-1}) after three days of storage under refrigeration (4±2°C).

The proposed method allows for the simultaneous identification and quantification of five biogenic amines by HPLC in chicken meat. The advantages were easy extraction, effective derivatization and high resolution in a short assay using an isocratic system of acetonitrile and water. This study demonstrated good selectivity, linearity, accuracy, recovery, robustness, LOD and LOQ. The method was successfully applied to chicken meat and showing that tyramine was at the highest levels in both commercial packages.

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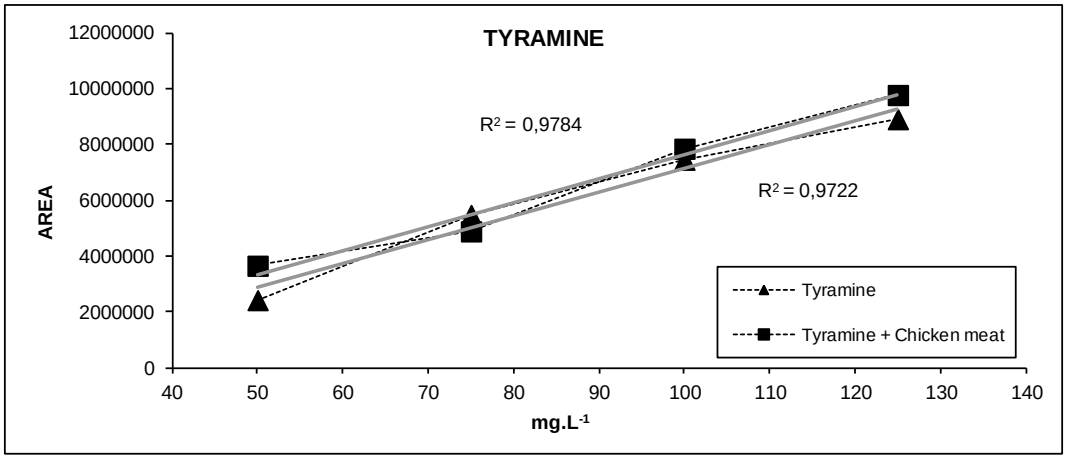
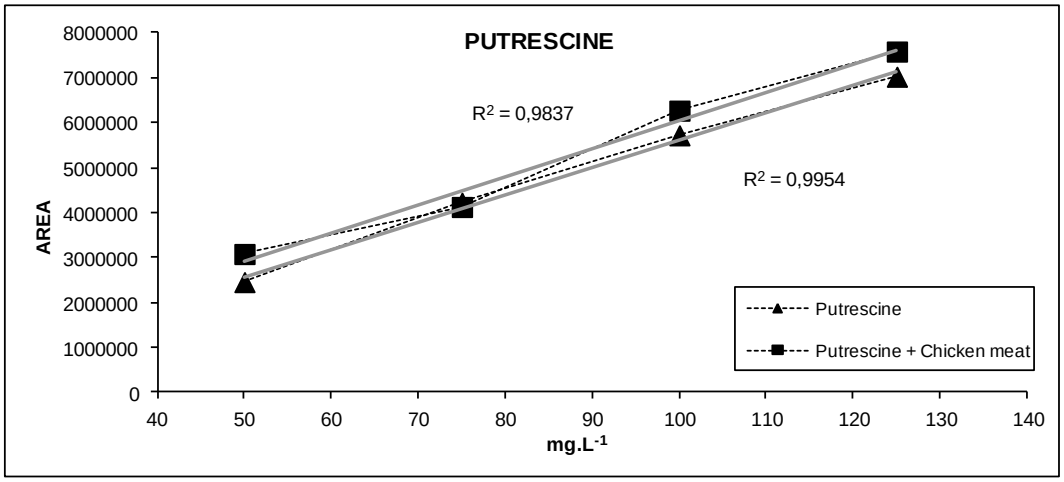
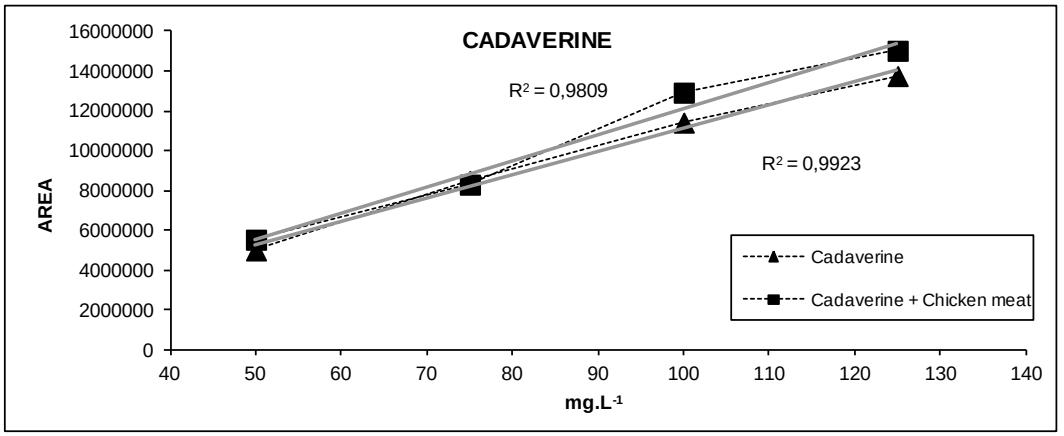
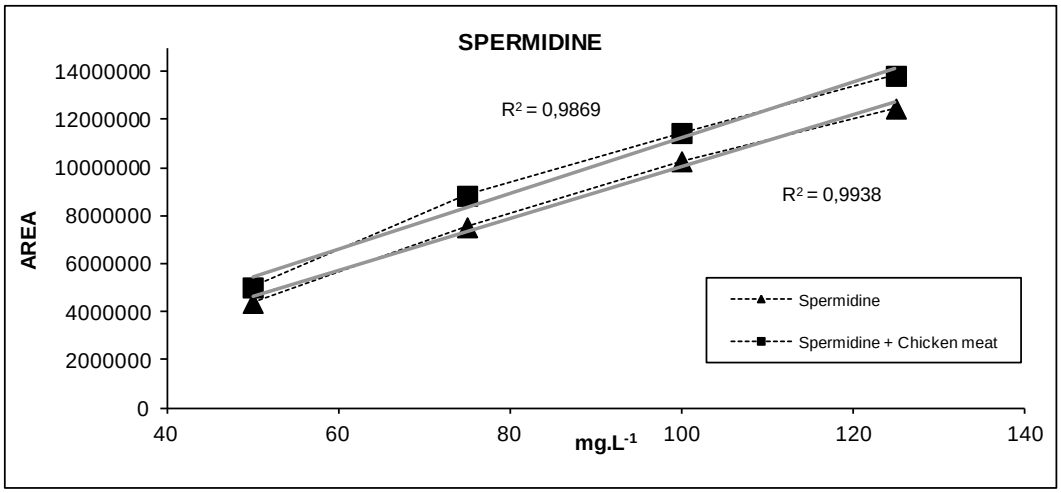

TYRAMINE
R² = 0,9784
R² = 0,9722
AREA
mg.L-1
Tyramine
Tyramine + Chicken meat


PUTRESCINE
R² = 0,9837
R² = 0,9954
AREA
mg.L-1
Putrescine
Putrescine + Chicken meat


CADAVERINE
R² = 0,9809
R² = 0,9923
AREA
mg.L-1
Cadaverine
Cadaverine + Chicken meat



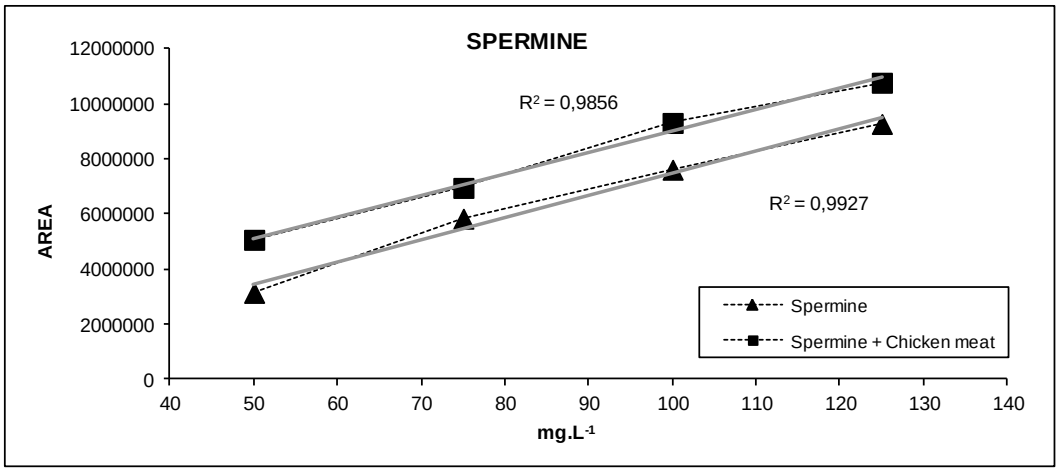


Fig. 1. Calibration curves for chicken meat samples spiked with amine standard and amine standard solution.

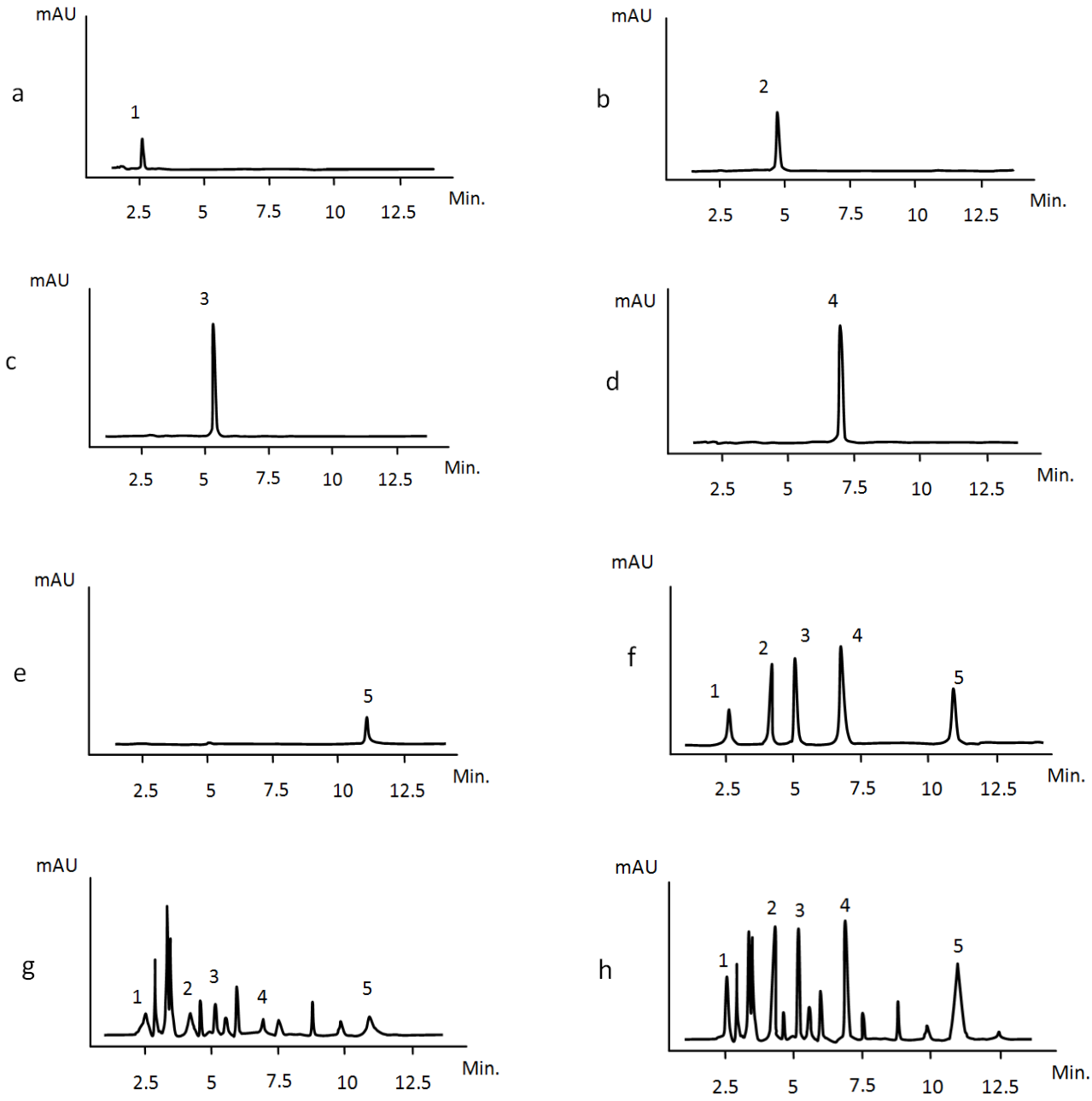


Fig. 2. Chromatograms of each amine standard (a, b, c, d, e), amine standard mixture (f), meat chicken sample (g) and meat chicken sample spiked with amine standard mixture (h). Peak identification: (1) tyramine, (2) putrescine, (3) cadaverine, (4) spermidine, and (5) spermine.

Table 1

Linearity of proposed chromatographic method for biogenic amine determination, obtained with eight different concentrations between 1 – 250 mg L^{-1}.

Biogenic amines	Regression equation	Regression coefficient (r^2)
Tyramine	Y = 26297X - 41220	0.9981
Putrescine	Y = 42896X + 16098	0.9977
Cadaverine	Y = 48731X + 43416	0.9997
Spermidine	Y = 72733X - 455722	0.9921
Spermine	Y = 44107X - 66297	0.9985

Table 2

Results of accuracy (repeatability) for the chromatographic method evaluated with three different concentrations of an amine standards mix solution.

Biogenic amines	Mix A			Mix B			Mix C		
	Mean ± SD	RSD	RT	Mean ± SD	RSD	RT	Mean ± SD	RSD	RT
Tyramine	315.81 ± 3.51	1.11	2.47	470.32 ± 5.78	1.23	2.50	1,038.51 ± 13.32	1.28	2.52
Putrescine	74.55 ± 1.07	1.43	4.36	167.60 ± 2.55	1.52	4.32	229.40 ± 0.59	0.26	4.38
Cadaverine	101.77 ± 2.47	2.43	5.29	201.09 ± 2.57	1.28	5.26	274.30 ± 5.30	1.93	5.24
Spermidine	82.86 ± 1.06	1.28	7.14	169.99 ± 1.53	0.90	7.12	230.65 ± 3.92	1.70	7.11
Spermine	92.67 ± 0.08	0.09	11.65	185.84 ± 2.70	1.45	11.68	227.80 ± 2.47	1.08	11.62

Mix A: Putrescine, cadaverine, spermidine and spermine (100 mg L^{-1}) + tyramine (300 mg L^{-1})
Mix B: Putrescine, cadaverine, spermidine and spermine (200 mg L^{-1}) + tyramine (500 mg L^{-1})
Mix C: Putrescine, cadaverine, spermidine and spermine (250 mg L^{-1}) + tyramine (1000 mg L^{-1})
SD = Standard deviation, RDS = Relative standard deviation, RT = Retention time

Table 3

Results of accuracy (intermediate precision) for the chromatographic method evaluated with the same amine standards mix solution on three different days.

Biogenic amines	Day 1			Day 2			Day 3		
	Mean ± SD	RSD	RT	Mean ± SD	RSD	RT	Mean ± SD	RSD	RT
Tyramine*	412.02 ± 6.76[a]	1.64	2.52[a]	415.52 ± 11.61[a]	2.79	2.46[a]	416.89 ± 30.18[a]	7.24	2.48[a]
Putrescine**	159.90 ± 2.50[a]	1.56	4.38[a]	159.03 ± 2.27[a]	1.43	4.34[a]	160.96 ± 2.74[a]	1.70	4.34[a]
Cadaverine**	178.42 ± 2.05[b]	1.15	5.30[a]	183.98 ± 2.27 [a]	1.23	5.26[a]	181.41 ± 5.67[a]	3.12	5.28[a]
Spermidine**	179.47 ± 9.44[a]	5.26	7.12[a]	180.98 ± 5.32[a]	2.94	7.10[a]	182.13 ± 6.45[a]	3.54	7.08[a]
Spermine**	172.70 ± 6.00 [a]	3.47	11.68[a]	168.04 ± 5.24[a]	3.12	11.60[a]	173.18 ± 5.17[a]	2.99	11.64[a]

* Concentration: 400 mg L^{-1}, ** Concentration: 200 mg L^{-1}

Different letters in rows represent significantly different averages ($P \leq 0.05$)

SD = Standard deviation, RDS = Relative standard deviation, RT = Retention time

Table 4

Recovery, limit of detection and limit of quantification for the proposed chromatographic method.

Biogenic amines	Recovery (%)			LOD ($\mu g\ L^{-1}$)	LOQ ($\mu g\ L^{-1}$)
	Mix A	Mix B	Mix C		
Tyramine	84.30	91.58	112.22	0.03	0.15
Putrescine	90.42	94.59	94.59	0.25	0.80
Cadaverine	73.43	106.54	111.16	1.25	5.00
Spermidine	81.33	91.27	99.43	0.25	0.78
Spermine	64.40	90.65	95.89	0.06	0.50

Mix A = 100 mg L^{-1} for each amine
Mix B = 150 mg L^{-1} for each amine
Mix C = 200 mg L^{-1} for each amine
LOD = Limit of detection
LOQ = Limit of quantification

Table 5

Results of robustness for variations in mobile phase concentration, wavelength, flow rate and derivatization time.

	Biogenic amines (mg L^{-1})				
Parameters	Tyramine	Putrescine	Cadaverine	Spermidine	Spermine
Acetonitrile:Water (v:v)					
41:59	407. 20 ± 6.97^{a}	180.98 ± 5.79^{a}	191.23 ± 9.18^{a}	165.86 ± 2.09^{a}	169.36 ± 6.67^{a}
42:58	411.29 ± 10.50^{a}	173.71 ± 4.80^{b}	201.16 ± 2.56^{a}	168.17 ± 2.42^{a}	170.28 ± 3.78^{a}
43:57	412.56 ± 7.88^{a}	174.69 ± 1.06^{b}	192.92 ± 7.73^{a}	165.02 ± 4.91^{a}	172.23 ± 7.50^{a}
Detector (nm)					
197	410.97 ± 6.33^{a}	176.37 ± 3.49^{a}	199.96 ± 1.49^{b}	176.86 ± 4.36^{a}	154.02 ± 2.36^{b}
198	410.75 ± 5.47^{a}	179.02 ± 5.21^{a}	210.40 ± 5.63^{a}	178.43 ± 3.45^{a}	167.35 ± 2.49^{a}
199	412.05 ± 4.14^{a}	173.73 ± 6.56^{a}	212.62 ± 3.43^{a}	172.53 ± 3.73^{a}	172.69 ± 6.11^{a}
Flow rate (mL.min^{-1})					
0.95	415.81 ± 6.66 ^{a}	180.31 ± 4.58^{a}	196.98 ± 4.97^{a}	179.99 ± 3.61^{a}	169.22 ± 0.70^{a}
1.00	402.29 ± 7.26^{ab}	184.24 ± 3.65^{a}	198.68 ± 4.92^{a}	176.92 ± 3.85^{a}	169.32 ± 1.44^{a}
1.05	397.38 ± 9.85^{b}	173.46 ± 1.64^{b}	197.73 ± 1.95^{a}	183.16 ± 1.36^{a}	169.51 ± 1.73^{a}
Time of derivatization (min)					
15	402.31 ± 5.43^{a}	159.63 ± 3.62^{b}	174.70 ± 5.81^{b}	184.06 ± 2.82^{b}	172.18 ± 4.91^{b}
20	409.68 ± 5.48^{a}	181.42 ± 6.03^{a}	195.94 ± 6.08^{a}	191.99 ± 5.47^{ab}	180.41 ± 1.12^{a}
25	408.56 ± 3.87^{a}	184.10 ± 2.05^{a}	194.82 ± 3.18^{a}	192.78 ± 2.30^{a}	186.01 ± 1.36^{a}

Results expressed as mean ± SD

Different letters in rows represent significantly different averages ($P \leq 0.05$)

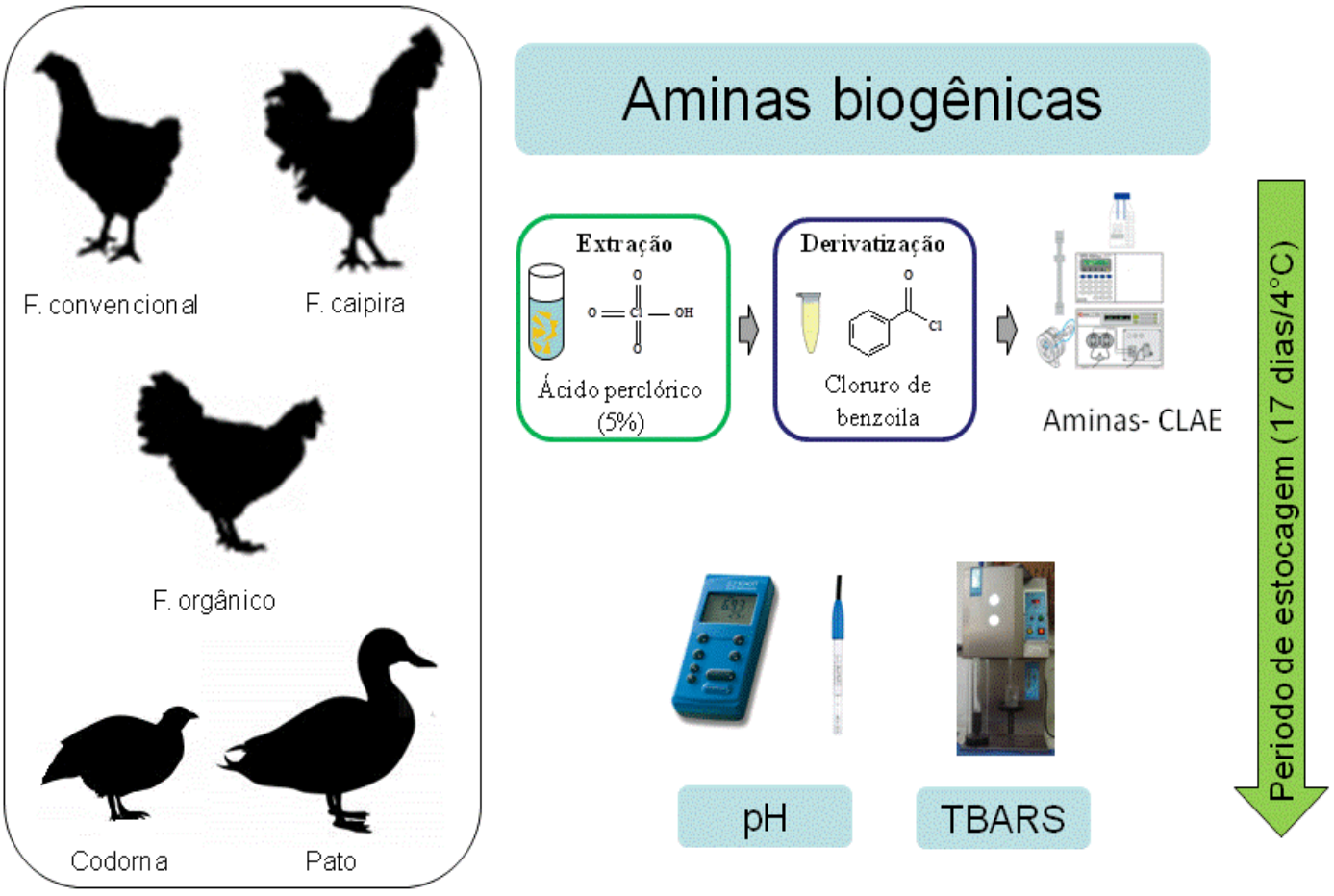
Table 6

Biogenic amine results from chicken breast meat stored in two commercial packages.

Biogenic amines	Package A			Package B		
	Mean ± SD	Range	RT	Mean ± SD	Range	RT
Tyramine	230.72 ± 78.07[a]	68.41 - 367.79	2.35	195.42 ± 40.26[b]	108.47 - 259.06	2.40
Putrescine	6.93 ± 2.59[a]	1.76 - 11.09	4.42	6.73 ± 3.20[a]	3.35 - 33.57	4.38
Cadaverine	4.71 ± 2.01[a]	1.37 - 8.96	5.28	4.57 ± 2.31[a]	1.18 - 9.73	5.30
Spermidine	9.80 ± 3.08[a]	6.40 - 15.94	7.10	10.77 ± 3.31[a]	6.33 - 18.84	7.17
Spermine	16.03 ± 4.02[a]	9.67 - 27.77	11.81	18.98 ± 5.26[a]	12.56 - 38.00	11.75

Different letters in rows represent significantly different averages ($P \leq 0.05$)
Package A: Chicken meat in expanded polystyrene tray. Package B: Chicken meat in plastic bag
RT: Retention time

3.2 ARTIGO 2: BIOCHEMICAL CHANGES IN ALTERNATIVE POULTRY MEAT DURING REFRIGERATED STORAGE. Published in Revista Brasileira de Ciência Veterinária (Paper II)



Biochemical changes in alternative poultry meat during refrigerated storage

Alterações bioquímicas em carne de aves alternativas durante o armazenamento em refrigeração

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Abstract

The aim of this study was evaluated the physical and chemical changes in five types of alternative poultry meat keep at refrigerated (4±1°C) conditions during 18 days. For this purpose, breast meat of conventional, organic and free-range chicken, duck and quail were purchase from markets of Niteroi city (Rio de Janeiro, Brazil). Levels of pH, thiobarbituric acid reactive substances (TBARS) and biogenic amines (cadaverine and putrescine) were determinate. The results showed a gradual and proportional increment of pH values (between 5.5 and 6.5) in all poultry meats during o storage time; an increment of TBARS values in the first days of storage, remaining stables to the end of experiment. A significant increment of putrescine values was observed after the sixth day remaining stable in conventional chicken and quail until the end of experiment while significant reduction was observed in the rest of poultry meats. Finally, only conventional and organic chicken and quail showed a gradual increment during storage time. Concluded that significant biochemical changes was observed during the storage time being that pH, cadaverine and putrescine values could be parameters using like deterioration indicators for these products.

Keywords: Biogenic amines, chicken, duck, pH, quail, quality, TBARS

Resumo

O objetivo do presente estudo foi avaliar as mudanças físico-químicas de cinco tipos de carnes de aves alternativas mantidas em condições de refrigeração (4±1°C) durante 18 dias. Para essa finalidade utilizou-se carne de peito de frangos convencional, caipira e orgânico, pato e codorna adquiridos de supermercados na cidade de Niterói (Rio de Janeiro, Brasil) e determinou-se os níveis de pH, substancias reativas ao acido tiobarbitúrico (SRATB) e aminas biogênicas (cadaverina e putrescina). Os resultados indicaram um incrementou gradativo e proporcional dos valores de pH (entre 5,5 e 6,5) nas carnes de aves durante o tempo de estocagem; um aumento dos valores de SRATB nos primeiros dias de estocagem, mantendo-se invariáveis até o final do experimento. Em todas as aves foi observado um incremento significativo dos valores de putrescina após o sexto dia, mantendo se estáveis no frango convencional e a codorna até o final do experimento enquanto o resto das carnes diminuiu significativamente. Finalmente, foi observado um incremento gradativo apenas na codorna e nos frangos convencional e orgânico. Conclui-se que houve mudanças bioquímicas significativas nas carnes avaliadas durante o tempo de estocagem, sendo que os valores de pH, cadaverina e putrescina seriam parâmetros que podem ser utilizados como indicadores de deterioração destes produtos.

Palavras-chave: aminas biogénicas, codorna, frango, pato, pH, qualidade, TBARS

Introduction

The Brazilian poultry industry sector of the contemporaneous economy, has undergone profound changes over the past 40 years, presenting a set of changes related to the organizational structure encompassing much of the production processes, administrative and work organization (Buzanello and Moro, 2012). Due to the increase global consumption of alternative meats, the poultry industry constantly searches different options to attend consumer demands. In Brazil, the alternative poultry production system was initially implemented by small and medium producers as an opportunity to offer differentiated products of higher quality providing producers higher income through added value in relation to intensive production system. However, in recent years the alternative production is widely implemented in the poultry industry (Santos et al., 2012).

The Brazilian free-range chicken system is regulated by the Brazilian legislation; according to that, the chicken named free-range is fed exclusively with plant origin ration and the use of growth promoters is prohibited. Also, is recommended that the birds are reared in extensive and three square meters of pasture per animal and the slaughter takes place at the minimum age of 85 days (Brasil, 1999).

An increasing number of consumers demanding healthier and naturally grown foods have favored organic live-stock farming, which is reputed to be environmentally friendly, allied with promotion of animals good health, with high welfare standards results in high quality products (Castellini et al., 2002)

The production of other types of meat poultry are also growing in the Brazilian market. Quail and duck production are becoming important because these birds do not require much space, are easy to manage and those productions are not expensive to implement (Carioni et al., 2001; Costa et al., 2012). All of these poultry meat are profitable alternatives for both small and medium farmers in a market with a great number of consumers willing to pay distinct prices for these products.

The poultry meat is a highly perishable product, susceptible to physical and chemical changes. Lipid oxidation is one of the principal chemical reactions occurring in muscle foods during refrigerated storage; it is initiated in the membrane-bound phospholipids which are susceptible to peroxidation due to their high concentration of long chain polyunsaturated fatty

acids (PUFA) (Higgins, 1998). Poultry meat is more susceptible to oxidative rancidity than red meat because of its higher content of PUFA (Pikul et al., 1984).

Biogenic amines are low molecular weight substances, formed mainly by decarboxylation of specific amino acids present in food through the action of bacterial enzymes during storage (Suzzi and Gardini, 2003). These substances have a great interest not only for public health on account of their toxicological effects but also by its use as a deterioration indicator (Silla Santos, 1996; Buňková et al., 2010).

The aim of this study was determinate the biochemical changes in alternative poultry meats and evaluated if these biochemical parameters could be used as a indicators of poultry meat deterioration.

Materials and methods

The present study evaluated the changes of pH, thiobarbituric acid reactive substances and biogenic amines (cadaverine and putrescine) in five types of poultry meat keep at refrigerated (4±1°C) conditions for 18 days.

A total of thirty breast meat (m. *Pectoralis maior*) of chicken (*Gallus gallus domesticus*) were divided equally on conventional, free-range and organic were used. Moreover, ten breast from duck (*Anas platyrhynchos domesticus*) and twenty from quail (*Coturnix coturnix*) were used. All samples were purchased from local markets in Niteroi (Rio de Janeiro, Brazil). Frozen samples were thawed overnight at 4±1°C and were aseptically cut into pieces of 100g, placed in plastic bags for refrigerated storage at 4±1°C for 18 days. In case of quail an entire breast of each carcass was used. Biochemical changes were evaluated every two days during 18 days on refrigeration storage.

All reagents used in the present study were of analytical grade. Cadaverine and putrescine standards (Sigma Aldrich, St. Louis, MO, USA) and 2-thiobarbituric acid (4,6-dihydroxy-2-mercaptopyrimidine) (Spectrum Chemical Mfg. Corp. New Jersey, USA) were purchased. Stock solution with 40µg of cadaverine and putrescine were prepared in 0.1*N* HCl and stored at 4±1°C. Different dilutions were performed and used for the calibration curve.

The progress of oxidation was determined by the thiobarbituric acid reactive substances (TBARS) test according to Tarladgis et al. (1960). In brief, 10g of sample were manually minced, homogenized with 97.5mL of distilled water and transferred to a distillation tube

which was added 2.5mL of HCl (4*N*). This sample was then distilled and the first 50mL of distillate was collected. Next, 5mL of the distillate were added to 5mL of 0.02M thiobarbituric acid and were heated in a boiling water bath (100°C) for 35 min for accelerate the reaction and, consequently, color development. The samples were immediately cooled with water and the absorbance was measured at 528 nm on a Smartspec Plus spectrophotometer (BioRad, Hercules, CA, USA). The final values were expressed as milligram of malondialdehyde (MDA) per kilograme. The pH values were performed by using a digital pH meter (Digimed® DM-22) equipped with a DME-R12 electrode (Digimed®) after briefly homogenization of 10 g of muscle sample with 90mL of distilled water (Conte-Júnior et al., 2008).

Biogenic amine determination was performed in accordance with a modified procedure proposed by Conte Junior et al. (2006). Extraction were carried out using 5g of meat and 5mL of perchloryc acid 5%; the mixture was homogenized (Certomat® MV, B. Braun Biotech International) for 30 seconds in ten minutes intervals up to one hour; the sample tubes were kept under refrigeration (4±2°C) during the whole process. The homogenate was centrifuged at 503*g* for 10min at 4±1°C (Hermle Z 360 K) and filtered with Whatman No. 1 paper. The filtrates were neutralized (pH>6) with 2*N* NaOH and kept in ice bath (0±1°C) for 20 min. A second filtration and alkalynization with NaOH (pH>12) were performed in the same conditions.

The derivatization of biogenic amines carried out with addition of 40μL benzoyl chloride, followed by homogenization in vortex for 15 sec and kept at room temperature (25±2°C) for 20 min. The biogenic amines were collected through liquid partitioning with 1000 μL of diethyl ether, which was proceeded in two times. The ether layers containing amines were evaporated to dryness under nitrogen stream (Sample Concentrator Techne, Cambridge, UK). Finally, the residue was dissolved in 1000μL of mobile phase (acetonitrile:water) and stored at 4±1°C.

The chromatographic system consisted of a Shimadzu LC/10AS liquid chromatography coupled to SPD/10AV UV-Vis detector and C-R6A Chromatopack integrator. Amines separation were performed on Teknokroma Tracer Extrasil ODS2 (15 x 0.46cm id., 5μm) column equipped with a Supelco, Ascentis C18 (2 x 0.40 cm, id. 5μm) guard column, in isocratic condition. The mobile phase was prepared by mixing acetonitrile (Tedia®) with ultrapure water purified on Millipore Simplicity (Millipore, Molsheim, France) at 42:58 (v/v);

and degassed in ultrasonic bath (Cleaner USC 2800 A). The chromatography conditions were: flow rate of 1 mL.min^{-1}, injection volume of 20µL, column temperature of 20°C and detector wavelength set at 198 nm. Injection was performed using a 50µL syringe (Hamilton TM 705) and total run time of 15 minutes. Injection of pure acetonitrile for 10 min was used between each sample for conditioning the HPLC system. Presence of biogenic amines were identified by retention time and quantified by peak area.

The statistic study of the pH, TBARS and biogenic amines content determined in different poultry meat was carried out with analysis of variance, and the means were compared with Tukey test ($p<0.05$). For the interpretation, data set was divided, for all above-mentioned analyses, in four periods: Period 0 (P0) – analysis of day 0 of storage; Period 1 (P1) – analysis of the first six days of storage; Period 2 (P2) – analysis of the subsequent six days; and Period 3 (P3) – analysis of the last six days of storage. GraphPad Prism® (Ver. 5.00., 2007) package for windows was used (Graphpad, 2007).

Results and discussion

At the beginning of the experiment quail, duck, free-range and conventional chicken had similar pH values between 5.57 and 5.77. On the other hand, organic chicken showed values above of 6.00. Through all of storage period all meats showed a significant increase ($p < 0.05$) demonstrating that pH can be considered a quality parameter (Figure 1). These results are similar to those found by Castellini et al. (2002) that determined pH values of 5.75 and 5.80 for organic chickens slaughtered at 56 and 81 days of age respectively; these values would be related to the rearing system of those birds in open environments and better welfare conditions which would reduce the stress pre-slaughter and further consumption of muscle glycogen. Free-range chicken had acidic values which are consistent with the results of Cheng et al. (2008) and Faria et al. (2009) who observed pH values between 5.70 and 5.90 after 24 h *post mortem* in free-range chicken, explaining that larger diameter muscle fibers was related to slow-growing chicken, which have higher glycolytic activity and reduced glycogen reserves before slaughter, fact that determine the final pH observed.

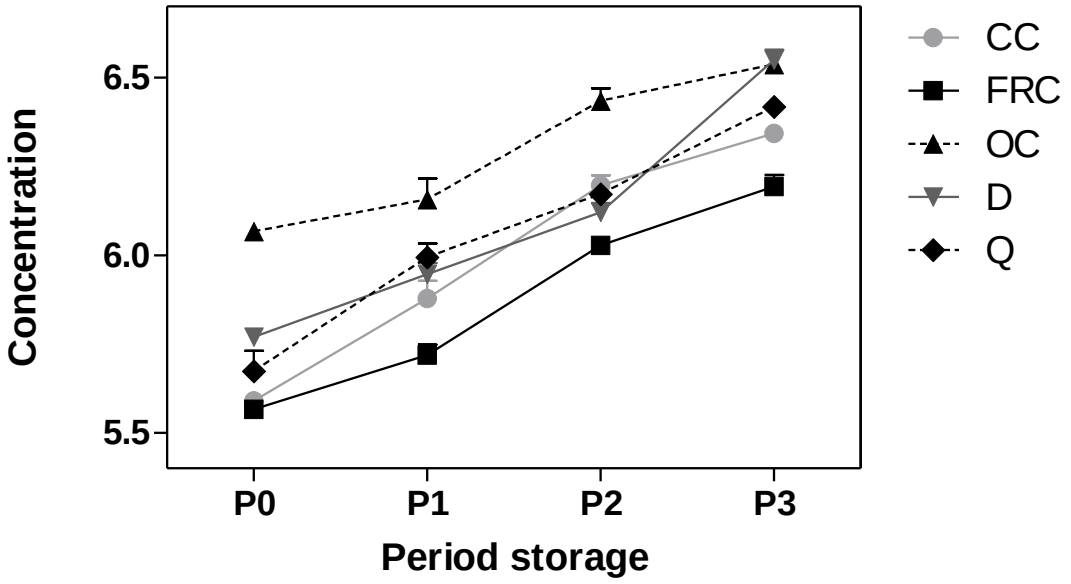

Figure 1. pH values in different poultry meats in refrigerated conditions (4 ± 1 °C) storage during 18 days. Abbreviations: CC = Conventional chicken, FRC = Free-range chicken, OC = Organic chicken, D = Duck, Q = Quail, P0 = Day 0 of storage; P1 = 1 – 6 days of storage, P2 = 7 - 12 days of storage, and P3 = 13 -18 days of storage

All poultry meats showed lower initial levels of TBARS (0.10–0.25mg.kg^{-1}). Conventional and free-range chicken meat showed no difference ($p<0.05$) during storage time (Figure 2). On the other hands, organic chicken, duck and quail showed a significant increase ($p>0.05$) in the first six days of storage and then these values remained stable until the end of the storage period; this fact suggest that a early increment of MDA in these birds indicate an early stage of rancidity, which would not be suitable as quality indicator like pH values. The quail showed highest values (above of 1mg.kg^{-1}) compared to other birds; these results can be explained because the quail were purchased with the skin that may have contributed to the increase of oxidative rancidity, while all chicken breasts were acquired without skin.

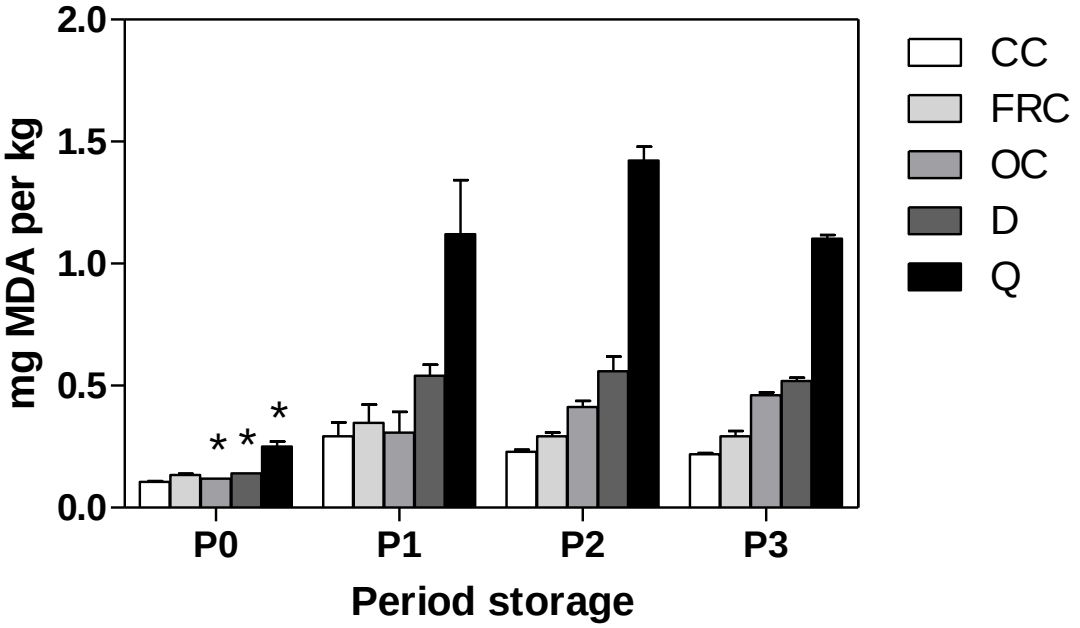

Figure 2. Thiobarbituric acid reactive substances (TBARS) concentration in different poultry meats in refrigerated conditions (4 ± 1 °C) storage during 18 days. The symbol * over the bars indicate significant differences between of periods storage in the same kind of poultry meat. Abbreviations: CC = Conventional chicken, FRC = Free-range chicken, OC = Organic chicken, D = Duck, Q = Quail, P0 = Day 0 of storage; P1 = 1 – 6 days of storage, P2 = 7 - 12 days of storage, and P3 = 13 -18 days of storage

Our results were similar to reported by Alasnier et al. (2000) who determined low initial levels of lipid oxidation 0.03 mg.kg^{-1} followed by a linear increment until 0.30 mg.kg^{-1} at day 14 in chicken breast meat; these authors suggested that lower ratios of vitamin E in chicken meat prevents the oxidation of long-chain PUFAs in the initials days and after the antioxidant reserves exhausted, the lipid oxidation is more evident. On the other hands, Castellini et al. (2002) and Castellini et al. (2006) evaluating the physical and chemical characteristics of organic chickens and found TBARS values above 2 mg.kg^{-1}, 24 h *post mortem*. They explained that the type of rearing of these birds, in open environments with intense muscle activity, could increase muscle oxidative metabolism and the production of free radicals. Husak et al. (2008) evaluated organic, free-range and conventional chickens, found TBARS values between 0.12 and 0.19 mg.kg^{-1}, not considered indicative of rancidity in any chicken.

Putrescine levels showed a gradually increase in conventional chicken and quail and after the first six days of storage it increased at significant levels ($p<0.05$). Similar behavior was observed in free-range chicken but the highest levels were detected between 7 and 12 day of storage, followed by a significant decrease in the last period of experiment. Organic chicken

showed a slightly increment between 7 and 12 day of storage but it was not significant ($p>0.05$). Duck levels also started at low level and significantly increased ($p<0.05$) between 2 and 12 day of storage; then it was observed a slightly decrease (Figure 3).

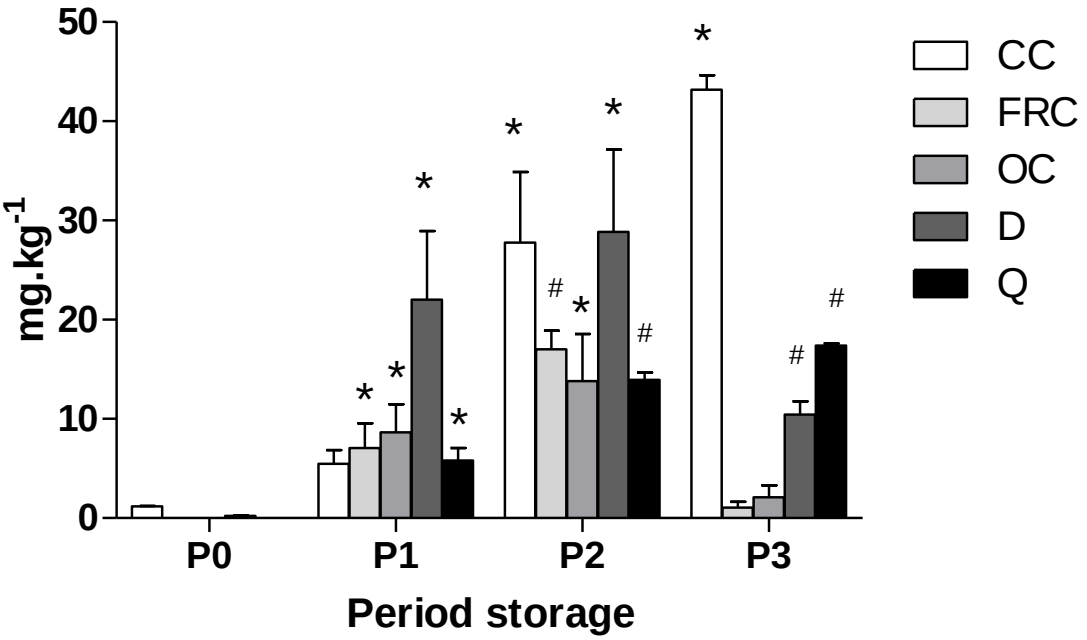

Figure 3. Putrescina concentration in different poultry meats in refrigerated conditions (4 ± 1 °C) storage during 18 days. The symbols (*,#) over the bars indicate significant differences between of periods storage in the same kind of poultry meat. Abbreviations: CC = Conventional chicken, FRC = Free-range chicken, OC = Organic chicken, D = Duck, Q = Quail, P0 = Day 0 of storage; P1 = 1 – 6 days of storage, P2 = 7 - 12 days of storage, and P3 = 13 -18 days of storage

Regarding, cadaverine it was observed that only organic meat showed detectable values at day 0. Conventional chicken showed the lowest values until the 12 day of storage when significantly increased ($p<0.05$). Free-range progressively increased until 12 day, after that a significantly decrease ($p<0.05$) was observed. Organic chickens and quail showed a gradually and significantly increase in all periods of storage. Duck values slightly increased in the first and then remain stables to the end of period storage ($p>0.05$) (Figure 4).

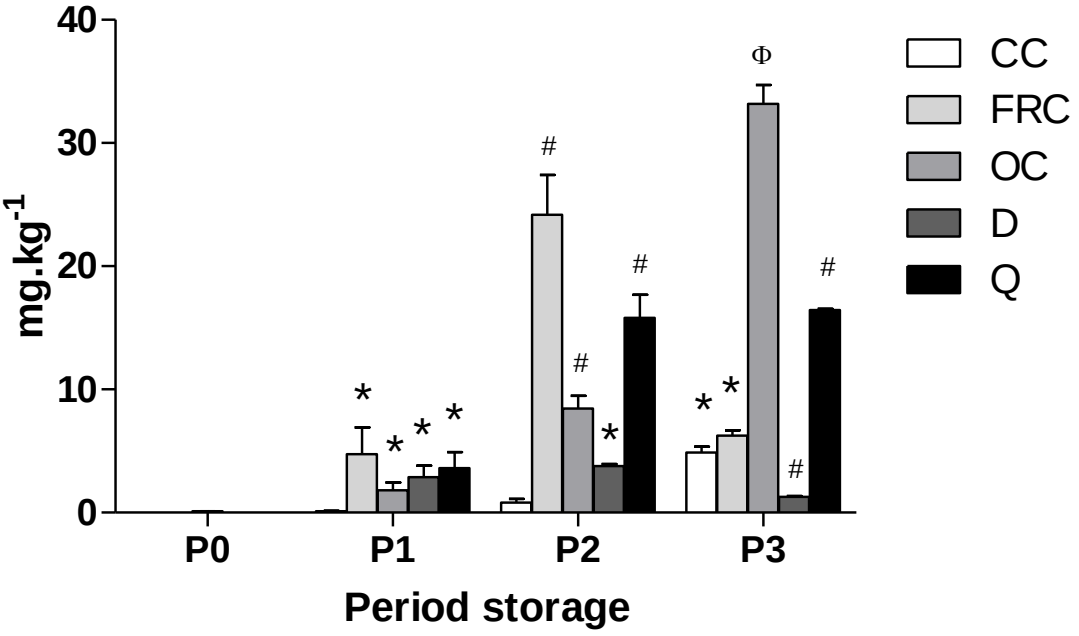

Figure 4. Cadaverina concentration in different poultry meats in refrigerated conditions (4 ± 1 °C) storage during 18 days. The symbols *, ϕ, # over the bars indicate significant differences between of periods storage in the same kind of poultry meat. Abbreviations: CC = Conventional chicken, FRC = Free-range chicken, OC = Organic chicken, D = Duck, Q = Quail, P0 = Day 0 of storage; P1 = 1 – 6 days of storage, P2 = 7 - 12 days of storage, and P3 = 13 -18 days of storage

Putrescine and cadaverine values were rather low in comparison to the levels reported by Sander et al. (1996), who found 200 and 500 mg.kg^{-1} respectively in chicken carcass 24 h *post mortem*. On the other hand, different authors described a gradually increase for those amines during storage. Silva and Gloria (2002) found 20.4 and 4.3 mg kg^{-1} for putrescine and cadaverine respectively in chicken meat storage at 4°C for 15 days; similar results were obtained by Rokka et al. (2004) for both amines in chicken meat storage at different temperatures between 2 and 8 °C; finally Balamatsia et al. (2007) who showed a linear increase from 53.8 to 409.6 mg.kg^{-1} and 19.8 to 252.8 mg.kg^{-1} for putrescine and cadaverine, respectively, in chicken meat storage at 4°C for 17 days. On the other hand, ours results of putrescine for duck meat were different to Dadáková et al. (2012) who determined values of 3.2 mg.kg^{-1} which slightly declined in the first week.

Vinci and Antonelli (2002) comparing biogenic amines, including putrescine and cadaverine, in beef and chicken stored at 4±1°C, reported that amines in white meat increases earlier than in red meat; they explained that it is due to shorter fibers in chicken than beef, which can be

easily attacked by proteolytic enzymes, resulting in the increased availability of amino acid precursors of biogenic amines. Tamim and Doerr (2003) determinated that levels of putrescine depends on the presence of ornithine, which in turn is produced from arginine degradation; consequently their formation may be ruled by arginine-utilizing microorganisms that produce ornithine as the substrate of decarboxylation. Ruiz-Capillas and Jiménez-Colmenero (2004) performed a compilation of several studies with regard to the behavior of amines in meat and meat by-products, showed that cadaverine and putrescine increased during storage.

Conclusion

The five poultry meat evaluation showed biochemical changes during the storage time. Values of pH and putrescine in all poultry and cadaverine in quail, organic and free-range chicken, had a significant increase and could serve as indicators of storage time after 12 days. TBARS did not seem to have relationship with the storage time because increase only took place in the first days. Further studies should be carried out to evaluated others biochemical indicators and the correlation with microbiological parameters.

Acknowledgments

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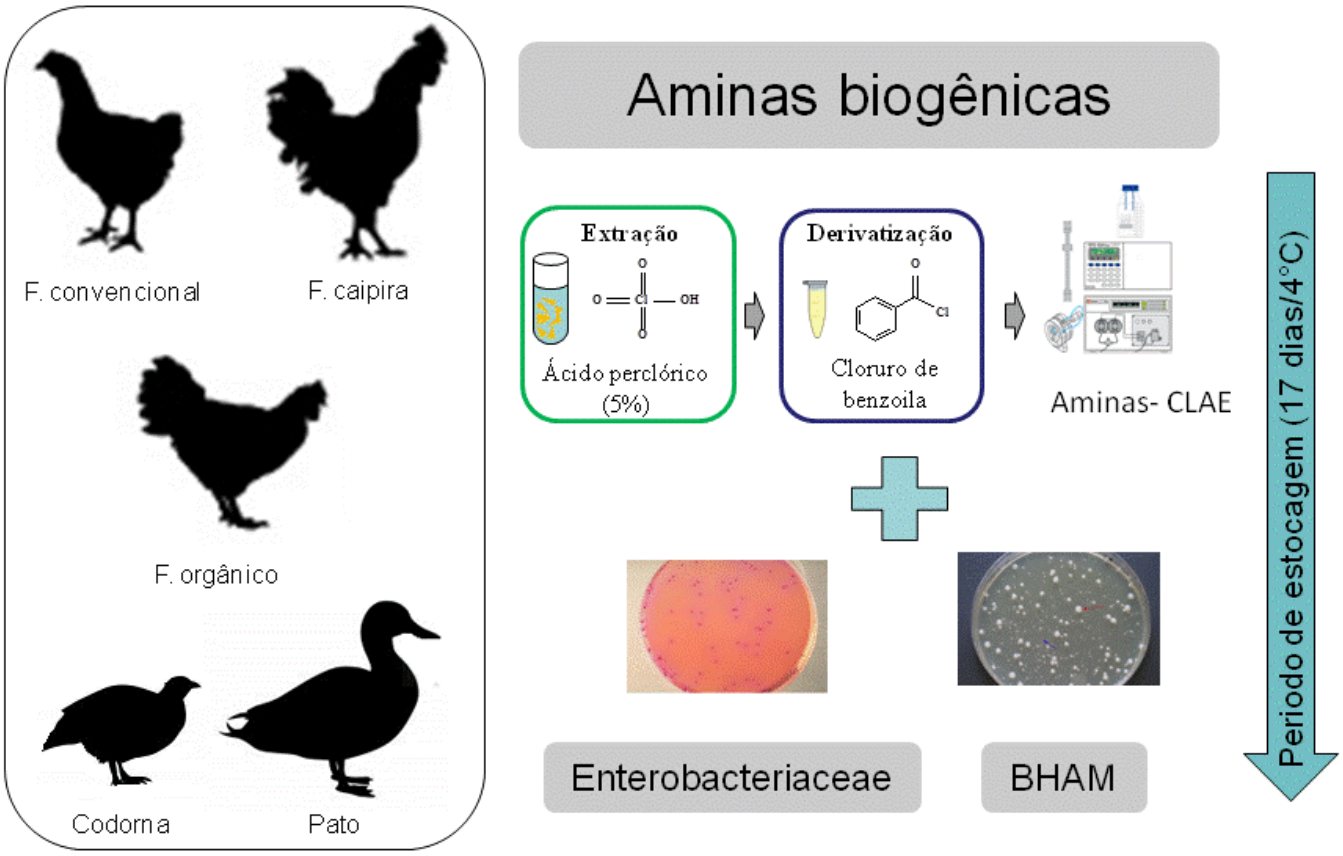
3.3 ARTIGO 3: BIOGENIC AMINES AS A BACTERIAL QUALITY INDICATOR IN DIFFERENT POULTRY MEAT SPECIES: A CHEMOMETRIC APPROACH. Submitted to Food Chemistry (Paper III)



BIOGENIC AMINES AS A BACTERIAL QUALITY INDICATOR IN DIFFERENT POULTRY MEAT SPECIES: A CHEMOMETRIC APPROACH

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Abstract

The production of biogenic amines and microbiology quality indicators (total mesophilic aerobic heterotrophic bacteria and Enterobacteriaceae counts) were evaluated in the breasts of five different poultry meats (duck and quail, as well as conventional, free-range, and organic chicken) during storage at 4±1°C for 17 days. Chemometric methods, such as the Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA), and supervised classification methods, such as the K-nearest neighbors (KNN) method and the soft independent modeling of class analogy (SIMCA), were used to discover possible relationships. The results suggest that there is a specificity of the biogenic amine profile depending on the poultry species. It was not possible to differentiate the samples adequately using PCA and HCA. In contrast, KNN and SIMCA were able to accurately identify 100% of the poultry meats, except for free-range chicken by SIMCA, of which only 50% of samples were identified. These findings suggest that biogenic amines, especially tyramine, putrescine and cadaverine, can be considered to be discrimination criteria among poultry meats.

Keywords: biogenic amines, poultry meat, chemometrics

1. Introduction

During the last three decades, the Brazilian poultry industry has experienced high growth rates. The flagship product, chicken meat, won the most demanding markets, distinguishing Brazil as the third largest producer in the world (Brasil, 2010). Due to the increase of global alternative meat consumption, the poultry industry has developed options that include free-range and organic chicken to address consumer demand (Brasil, 1999, 2008). Recently, other poultry species such as duck and quail have also gained relative importance due to their unique characteristics, including robustness, easy handling, inexpensive implementation, and the small space needed to raise them, contributing to the diversification of alternative poultry meats (Carioni, Porto, Padilha, & Sant'anna, 2001; Oliveira, Almeida, Mendes, Roça, & Veiga, 2005).

The consumption of these products, considered alternatives to conventional chicken meat, is gaining popularity due mainly to their sensory attributes and the fact that these meats are considered healthier than red meat (Balamatsia, Paleologos, Kontominas, & Savvaidis, 2006). However, the poultry meat is a highly perishable product that is susceptible to physical, chemical and microbiological changes; as a result, the shelf-life reduction is expressive during the first week of refrigerated storage (Balamatsia, Patsias, Kontominas, & Savvaidis, 2007). The quality and freshness of poultry meat are based mainly on the determination of sensory and microbial attributes. However, due to the lack of a general agreement as to the early signs of poultry meat spoilage and the subjectivity of sensorial perception, a number of chemical indicators, including variation in pH values, oxidative rancidity and levels of biogenic amines, have been proposed to assess the quality of poultry meat (Balamatsia, Paleologos, Kontominas, & Savvaidis, 2006).

Biogenic amines are low-molecular-weight substances formed mainly by the decarboxylation of specific amino acids present in food through the action of bacterial enzymes during storage (Silla Santos, 1996); consequently, the total amount and variety of amines formed strongly depends on the nature of the food and on the microorganisms present. Therefore, biogenic amines can be used as an indicator of microbial development (Min, Lee, Jang, Lee, & Kim, 2004; Vinci & Antonelli, 2002). Many Enterobacteriaceae and certain lactobacilli (e.g., *Lactobacillus buchneri*), pediococci and enterococci are particularly active in the formation of biogenic amines (Halász, Baráth, Simon-Sarkadi, & Holzapfel, 1994). There is great interest in biogenic amine detection in food, not only because these compounds

represent a public health risk on account of their toxicological effects related to the consumption of products with high concentrations of some amines (e.g. tyramine, histamine) but also for their use as a bacterial quality indicator (Silla Santos, 1996).

The aim of this study was to determinate the relationship between the biogenic amine levels and the microbiological changes in different poultry meat species using chemometric methods. We also evaluated the usefulness of these compounds as indicators of bacterial quality in five types of poultry meat stored at 4±1 °C for 17 days.

2. Materials and Methods

2.1. Meat samples

A total of forty poultry breast meats (m. *Pectoralis major*), divided equally among conventional (CONV), free-range (FREE) and organic (ORG) chicken (*Gallus gallus domesticus*) and duck (DU) (*Anas platyrhynchos domesticus*) and twenty carcass of quail (QUAL) (*Coturnix coturnix*) were purchased from local markets in Niteroi (Rio de Janeiro, Brazil). Frozen samples were thawed overnight at 4±1°C in their original packages and then aseptically removed from their containers. Ten breasts of each matrix (duck and organic, free-range and conventional chicken) were randomly divided into 100 g portions and placed aseptically into plastic bags (n=20). Due to the smaller size of quail breasts in relation to those of other birds, the whole breast (100 g) of each quail was used for each analysis (n=20). All samples were kept refrigerated at 4±1°C for 17 days.

2.2. Reagents and standards

All reagents used in the present study were of analytical grade. The biogenic amine standards were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Stock solutions with 40 µg cadaverine, putrescine, tyramine, spermidine and spermine were prepared in 0.1 N HCl and stored at 4±1 °C. Different dilutions were prepared and used for calibration curve construction, limits of detection and quantification and recovery of the proposed method.

2.3. Biogenic amine determination

Biogenic amine changes were evaluated every two days during refrigeration. The meat samples were processed in accordance with a modified procedure proposed by Conte Junior, Hierro, and Alvarez (2006). The method was validated for linearity, LOD, LOQ and recovery.

The extraction and derivatization were carried out with 5 g minced chicken breast meat. The homogenization was performed with 5 mL perchloric acid 5%, which was refrigerated (4±2°C) for 1 h and shaken continuously (Certomat® MV, B. Braun Biotech International, Melsungen, Germany); then, the mixture was centrifuged at 503×*g* for 10 minutes at 4±1 °C (Hermle Z 360 K, Wehingen, Germany) and filtered through Whatman No. 1 filter paper. The filtrates were neutralized (pH > 6) with 2 *N* NaOH and kept in an ice bath (0±1°C) for approximately 20 minutes, after which a second filtration and addition of NaOH (pH > 12) were performed under the same conditions. A strongly alkaline medium was prepared to improve the derivatization (Morgan, 1998).

The derivatization was performed with the addition of benzoyl chloride (40 μL), followed by homogenization via vortexing for 15 seconds and maintenance at room temperature for 20 minutes. The mixture was then extracted two times with 1000 μL diethyl ether. The ether layer was aspirated and evaporated to dryness under a stream of nitrogen (Sample Concentrator Techne®, Cambridge, UK). Finally, the residue was dissolved in 1000 μL mobile phase (acetonitrile:water) and stored at 4±1°C.

The chromatographic system consisted of a Shimadzu LC/10AS liquid chromatography coupled to a SPD/10AV UV-Vis detector and C-R6A Chromatopac Integrator (Shimadzu, Kyoto, Japan). The amine separation was performed on a Teknokroma Tracer Extrasil ODS2 (15 x 0.46 cm ID, 5 μm) column equipped with a Supelco Ascentis C18 (2 x 0.40 cm ID, 5 μm) guard column under isocratic conditions. The mobile phase was prepared by mixing acetonitrile (Tedia®) and ultrapure water purified with a Millipore Simplicity system (Millipore, Molsheim, France) at a ratio of 42:58 (v/v) and degassed in an ultrasonic bath (Unique Cleaner USC 2800 A, São Paulo, Brazil). The chromatography conditions were as follows: a flow rate of 1 mL min^{-1}, an injection volume of 20 μL, and a column temperature of 20°C. Benzoylated polyamines were detected by UV absorption at 198 nm because the absorbance has been shown to increase by approximately 50 times when acetonitrile is used as a solvent (Ali, Poortvliet, Strömberg, & Yngve, 2011). Each sample was injected two times using a 50 μL syringe (Hamilton Microliter TM 705, Reno, NV, USA) with a total run time of 15 minutes. An injection of pure acetonitrile for 10 minutes was used between each pair of samples to clean the HPLC system. The biogenic amines that were present were identified by the retention time and quantified by the peak area.

2.4. Bacteriological tests

The bacteriological analyses were performed in duplicate under laboratorial conditions simultaneously with the biogenic amines analysis. A 25 g aliquot of each sample was transferred aseptically to sterile homogenization bags with 225 ml 0.1% peptone water, and serial dilutions (10^{-2} to 10^{-5}) were prepared (Álvarez-Astorga et al. 2002). The total aerobic mesophilic bacteria (TAMB) were enumerated on Plate Count Agar (PCA) by the pour-plating method, followed by incubation at 35°C for 48 h (APHA, 1998). For the Enterobacteriaceae count, serial dilutions were pour-plated in Violet Red Bile Glucose Agar (VRBGA) with an overlayer and incubated at 35°C for 48 h. After the incubation period, the bacterial colonies were counted using a Quebec colony counter (Leica, Inc., Buffalo, USA) (Leite & Franco, 2009). The total count results were transformed into Log CFU g^{-1}.

2.5. Statistical analysis

The statistical studies of the biogenic amine contents determined in different poultry meat species and their relation to the time of storage were carried out by analysis of variance (ANOVA). The means were compared with Tukey's test ($p < 0.05$), and Pearson's analyses were performed for the correlation of microbiological and biogenic amines. The GraphPad Prism® (Ver. 5.00., 2007) package for Windows was used in these analyses.

Chemometric analyses (PCA, SIMCA and KNN) were performed using the PIROUETTE 2.2 software (Infometrix, Seattle, WA) while HCA was performed using XLSTAT version 2012.5 (Addinsoft, Paris, France). The dataset consisted of a 180 x 8 matrix in which rows represented the poultry meat species and columns represented the response variables of the biogenic amines (cadaverine, putrescine, tyramine, spermidine and spermine) and the microbiological indicators (total mesophilic aerobic heterotrophic bacteria and Enterobacteriaceae counts). These methods have been used in different processed foods and beverages such as beer (Granato, Branco, Faria, & Cruz, 2011), milk (Souza, Cruz, Walter, Faria, Celeghini, Ferreira, et al., 2011) and yogurt (Cruz, Cadena, Alvaro, Sant'Ana, Oliveira, Faria, et al., 2013).

The classification rules for the supervised chemometric techniques were validated by dividing the complete data set into a training set and an evaluation set. Samples were assigned randomly to the training set, which included 75% of the samples, and the test set, composed of the remaining 25% of the samples. These percentages are sufficient to perform this study. All data were auto-scaled before the analysis; that is, each column data matrix was mean-centered and scaled to the unit variance.

3. Results and discussion

The validation results showed good linearity for all biogenic amine standards (r^2 > 0.99). The LODs were 0.03, 0.25, 1.25, 0.25, and 0.06 µg L^{-1}, and the LOQs were 0.15, 0.80, 5.00, 0.78, and 0.50 µg L^{-1} for tyramine, putrescine, cadaverine, spermidine and spermine, respectively; the recovery was between 90 and 106% for all amines. Figure 1 shows the separation and identification of each biogenic amine in the standard solution and the chicken meat sample.

3.1. Biogenic amines

The results of the biogenic amine levels during storage can be observed in Table 1. Among all the amines examined in the present study, tyramine was the most abundant. At the beginning of the experiment, quail and organic chicken showed the highest (356.79 mg kg^{-1}) and the lowest values (96.55 mg kg^{-1}) of this amine, respectively; subsequently, these values showed a significant reduction in all meats ($p < 0.05$). Ours results were higher than those of Vinci and Antonelli (2002) and Balamatsia, Paleologos, Kontominas, and Savvaidis (2006), who reported lower values for tyramine (between 10 and 40 mg kg^{-1}) in chicken meat stored for different periods. Nassar and Emam (2002) suggested that high tyramine levels are mainly related to the presence of certain bacteria with an activated tyrosine decarboxylase enzyme that converts tyrosine into tyramine. Bover-Cid and Holzapfel (1999) reported that lactic acid bacteria strains such as *Lactobacillus brevis, L. buchneri, L. curvatus, Carnobacterium* spp., and *Enterococcus* spp. had the most intensive tyrosine decarboxylation activity; however, this bacterial group was not identified in our study due to its high relation to fermented products. In another study, Patsias, Chouliara, Paleologos, Savvaidis, and Kontominas (2006) evaluated the relation between biogenic amines and microbial changes in precooked chicken meat, in which they observed lower values of tyramine. From this observation the authors suggested that a study of the capability of microorganisms present to decarboxylate tyrosine would be appropriate.

The levels of putrescine increased significantly in conventional chicken and quail ($p < 0.05$), reaching 45.29 and 17.72 mg kg^{-1} at the end of experiment, respectively. The same behavior was observed in free-range chicken, organic chicken and duck, reaching 20.23, 28.68 and 54.82 mg kg^{-1}, respectively, between days 9 and 11, followed by a decrease. The cadaverine levels in free-range and organic chickens progressively increased to approximately 33 mg kg^{-1} on days 11 and 15, respectively; after that, a gradual decrease until the end of

experiment was observed only in free-range chicken. The cadaverine levels in the quail meat also increased, reaching 19.65 mg kg^{-1} on day 11, followed by a decrease to 16.33 mg kg^{-1} at the end of the experiment. Conventional chicken showed the lowest values for cadaverine, with 0.09 mg kg^{-1} on day 3, progressively increasing to 5.69 mg kg^{-1} at the end of experiment.

The putrescine and cadaverine values were rather low in comparison to the levels reported by Sander, Cai, Dale, and Bennett (1996), who found 200 and 500 mg kg^{-1}, respectively, in chicken carcasses 24 h *post mortem*. Other authors have described a gradual increase in those amines during storage. Silva and Gloria (2002) found 20.4 and 4.3 mg kg^{-1} for putrescine and cadaverine values, respectively, in chicken meat stored at 4°C for 15 days; similar results were obtained by Rokka, Eerola, Smolander, Alakomi, and Ahvenainen (2004) for both amines in chicken meat stored between 2 and 8°C; finally Balamatsia, Paleologos, Kontominas, and Savvaidis (2006) showed a linear increase of 53.8 to 409.6 mg kg^{-1} and 19.8 to 252.8 mg kg^{-1} for putrescine and cadaverine levels, respectively, in chicken meat stored at 4°C for 17 days. Our results regarding the levels of putrescine in duck meat differed from those reported by Dadáková, Pelikánová, and Kalač (2012), who measured a value of 3.2 mg kg^{-1}, which declined slightly in the first week.

Levels of putrescine depend on the presence of ornithine, which, in turn, is produced from arginine; consequently, their formation may be conditioned by arginine-utilizing microorganisms that produce ornithine as the substrate for decarboxylation. Ornithine is not one of the amino acids incorporated in proteins, and proteolysis does not result in an increased level of ornithine (Tamim & Doerr, 2003). In this context, the availability of free ornithine apparently limits the amount of putrescine. Bover-Cid, Miguelez-Arrizado, Luz Latorre Moratalla, and Vidal Carou (2006) indicated the relationship between putrescine and a wide spectrum of spoilage microorganisms.

The spermidine levels in all meats had initial values between 6.77 and 7.78 mg kg^{-1}. Only conventional chicken showed a slight increase in the first 5 days, followed by a gradual decrease. By day 9, all meats showed values between 6.29 and 6.57 mg kg^{-1}, respectively, which remained constant ($p > 0.05$) until the end of storage. In the case of spermine, the initial values were between 8.98 and 10.38 mg kg^{-1} for all meats except for that of organic chicken, which contained 23.67 mg kg^{-1}. After 9 day, there was a marked reduction of all values, reaching ca. 1.50 mg kg^{-1} and remaining constant for the rest of storage ($p > 0.05$).

The spermine and spermidine levels were similar and constant in all poultry meats because these polyamines occur naturally in fresh meat and are found at relatively constant levels (Hernández-Jover, Izquierdo-Pulido, Veciana-Nogués, Mariné-Font, & Vidal-Carou, 1997). These results are similar to those reported in other studies of chicken meat. Silva and Gloria (2002) found that spermine and spermidine showed values of 7.3 and 17.9 mg kg^{-1}, respectively, during the first days of storage at 4±1°C. Rokka, Eerola, Smolander, Alakomi, and Ahvenainen (2004) determined that the values of both polyamines remained constant for the first 12 days of storage. Balamatsia, Paleologos, Kontominas, and Savvaidis (2006) demonstrated that the initial values of spermine (approximately > 50 mg kg^{-1}) decreased during storage, and they suggested that these two amines cannot be used as indicators of fresh chicken meat quality. Our results regarding duck meat were similar to those of Dadáková, Pelikánová, and Kalač (2012) for spermidine and spermine.

The behavior of the biogenic amines observed in this study has been partially described in previous studies. Tamim and Doerr (2003) evaluated the effect of putrefaction on biogenic amine levels in chicken carcasses and observed that tyramine, putrescine and cadaverine increased 24 h *post mortem*, and spermine and spermidine remained constant. Ruiz-Capillas and Jiménez-Colmenero (2004) compiled several studies regarding the behavior of amines in meat and meat by-products and demonstrated that tyramine, cadaverine and putrescine increased during storage; however, these authors commented that spermine and spermidine had a tendency to decrease. Jastrzębska (2012) studied the behavior of different biogenic amines in chicken meat stored at 4°C for 5 days and determined that histamine, putrescine, cadaverine, and tyramine are the main amines formed during storage and that they increase with storage time. Vinci and Antonelli (2002) reported that all amines in chicken meat increased earlier than in beef stored at 4±1°C. They explained that this difference is likely due to the shorter fibers of chicken muscles, which can be easily attacked by proteolytic enzymes, resulting in increased availability of the amino acid precursors of biogenic amines.

3.2. Total aerobic mesophilic bacteria

All meats showed initial values of approximately 3 Log CFU g^{-1} for TAMB. These values gradually increased to approximately 7 Log CFU g^{-1} between days 7 and 11, except for quail, which reached 6.95 Log CFU g^{-1} at the end of experiment (Table 2). Senter, Arnold, and Chew (2000) established 7 Log CFU g^{-1} as the limit of microbial quality for mesophilic bacteria. Using this value, our results demonstrated that all meats were characterized by a

good bacteriological condition until day 9, except for organic chicken, which exceed this level at day 7. These results are in agreement with earlier reports of refrigerated chicken meat. Álvarez-Astorga et al., (2002) evaluated bacterial conditions in different markets and found values of 5.8 Log CFU g^{-1}; they attributed this level of meat contamination to inappropriate handling. Smolander, Alakomi, Ritvanen, Vainionpää, and Ahvenainen (2004) and Balamatsia, Paleologos, Kontominas, and Savvaidis (2006) found initial values of 4.9 and 4.0 Log CFU g^{-1}, respectively, in chicken meat, which gradually increased to 7 Log CFU g^{-1} by day 15.

3.3. Enterobacteriaceae

All meats had initial values between 2.00 and 3.60 Log CFU g^{-1}. These values began to increase gradually to levels above 7 Log CFU g^{-1} at days 7 and 9 in organic and free-range chicken, respectively. Conventional chicken, duck and quail did not exceeded this level during the experiment but reached very similar values at different times during storage (Table 2). Our results agree with the findings of Smolander, Alakomi, Ritvanen, Vainionpää, and Ahvenainen (2004), who found values below 7 Log CFU g^{-1} in chicken meat after storage at 4±1°C for 12 days.

Enterobacteriaceae are microorganisms that are generally considered to have a high decarboxylase activity; consequently, they have been associated principally with the production of putrescine and cadaverine (Curiel, Ruiz-Capillas, Rivas, Carrascosa, Jiménez-Colmenero, & Muñoz, 2011). Durlu-Özkaya, Ayhan, and Vural (2001) observed that the major biogenic amines produced by bacteria of this group were cadaverine, putrescine and tyramine in culture medium supplemented with specific amino acids and ground meat and hamburger. According to Rokka, Eerola, Smolander, Alakomi, and Ahvenainen (2004), the formation of biogenic amines is related to the amounts of certain microorganisms, particularly Enterobacteriaceae, which may be part of the original microbiota in food products or may be introduced by contamination during food processing. More recently, Buňková, Buňka, Klčovská, Mrkvička, Doležalová, and Kráčmar (2010) explored the production of biogenic amines by Gram-negative bacteria isolated from poultry skin. They found that Enterobacteriaceae strains were the largest producers of putrescine and cadaverine. The formation of biogenic amines from Enterobacteriaceae was also observed in other products. Marino, Maifreni, Moret, and Rondinini (2000) found a positive correlation between the concentration of cadaverine and Enterobacteriaceae counts in cheese; the same bacterial group

was identified by Curiel, Ruiz-Capillas, Rivas, Carrascosa, Jiménez-Colmenero, and Muñoz (2011) as being responsible for the production of putrescine and cadaverine in pig meat.

3.4. Relationship between biogenic amines and bacterial growth

Overall, the biogenic amines showed different trends during the storage of each type of poultry meat; in contrast, the counts of Enterobacteriaceae and TAMB tended to increase with storage time. The results of the Pearson correlation test (Table 3) showed that the relationships between bacterial groups and some amines depend on the type of poultry meat tested. With the exception of free-range chicken, both bacterial groups in all poultry meats showed a high negative correlation with tyramine values ($p < 0.05$). Free-range chicken and quail showed a high positive correlation between both bacterial groups and putrescine ($p < 0.05$) and cadaverine ($p < 0.05$). Finally, the bacterial results in all poultry meats showed a negative correlation for spermine and spermidine values ($p < 0.05$). These results support the relationship between the growth of Enterobacteriaceae and the TAMB results for the production of certain biogenic amines. There is no clear tendency for the use of certain amines as indicators of meat quality; on the contrary, it seems that each type of poultry meat presents an independent behavior with regard to the levels of amines during storage

There is very little information correlating biogenic amines and bacteriological growth in fresh poultry meat. Rokka, Eerola, Smolander, Alakomi, and Ahvenainen (2004) and Balamatsia, Paleologos, Kontominas, and Savvaidis (2006) observed a strong positive correlation between Enterobacteriaceae and TAMB putrescine, cadaverine and tyramine in chicken meat. This correlation was also observed in other meat products. Daher and Simard (1985) found a high correlation ($p < 0.05$) between TAMB and some amines (putrescine, cadaverine, tyramine and spermidine) in beef. Veciana-Nogués, Mariné-Font, and Vidal-Carou (1997) observed a relationship between tyramine and cadaverine with TAMB, Enterobacteriaceae and coliforms in refrigerated tuna meat. Nassar and Emam (2002) studied biogenic amine formation in chicken meat products and found a positive correlation between putrescine and Enterobacteriaceae. Patsias, Chouliara, Paleologos, Savvaidis, and Kontominas (2006) determined that putrescine and tyramine levels may be used to indicate the limit for spoilage initiation in precooked chicken meat stored aerobically for 8 days; in this time, the TAMB count was 6.5 log CFU g^{-1}.

3.5. Chemometric Techniques

The results of the PCA performed on the scores of the bacterial and biogenic amine parameters are shown in Figure 2a. Factor loading and observation scores are plotted together. The first two principal components explained 70.49% of the overall variability, while PCA 1 explained 57.66% and PC2 explained 12.86%. However, the results indicate that poultry meat cannot be clearly differentiated by species, suggesting that the parameters (biogenic amines and bacteriological counts) are not suitable for distinguishing between meats. The same behavior can be observed in the HCA dendrogram (Figure 2b), where all of the samples could be grouped into three clusters. However, the poultry meats are grouped together. Given that PCA and HCA are not suitable methods for data classification, we have suggested other methods to determine this information.

In this context, the SIMCA and KNN chemometric techniques were evaluated. Table 4 presents the recognition and prediction abilities afforded by each chemometric technique. KNN achieved recognition and prediction percentages of 100% for both poultry meat species, while the SIMCA report was inadequate for the FREE category, with a value of 50% in the prediction step. A total of 100% sensitivity and specificity were reported in all chemometric techniques adopted, except for the FREE classes in the SIMCA technique, for which these values were 62% and 45% in the prediction step, respectively. Putrescine, cadaverine and tyramine were the variables with the most discriminative power in the SIMCA technique, with values of 87, 50.6 and 47, respectively (data not shown). Our findings may be useful because this HPLC technique for biogenic amine determination requires less time, approximately 3 h for extraction and derivatization and 20 min running time in the HPLC instrument with real-time results. In contrast, the bacterial analyses require the preparation of a culture medium in advance, particular temperature conditions and at least 48 h of bacterial growth, and these conditions may change depending on the bacterial group. The HPLC technique could be an important advantage in industry due to the need for faster and more efficient methods of quality control.

However, some additional considerations should be introduced. The first consideration is the amino acid and protein content of poultry meats. Husak, Sebranek, and Bregendahl (2008) found a higher protein content in free-range organic chicken compared with conventional chicken. Another consideration is the type of microorganisms present in the meat. Nassar and Emam (2002) observed that the levels of biogenic amines (histamine, tyramine, cadaverine and putrescine) varied in the different chicken meat products examined; according to these authors, this variation could be attributed to differences in the

manufacturing process applied to each product. In this case, it is possible that poultry meat can be contaminated by different microorganisms. Halász, Baráth, Simon-Sarkadi, and Holzapfel (1994) determined that not all pathogenic or meat-spoilage bacteria produce decarboxylases that yield putrescine. In our results, the processing of each type of poultry meat was not controlled.

4. Conclusion

After the evaluation of the bacterial quality and the identification of the biogenic amines in poultry meat, we can conclude that the five poultry meats behaved differently with regard to the biogenic amine values during storage and that tyramine, putrescine and cadaverine are the major biogenic amines that can be used as indirect bacterial indicators. These results were confirmed by chemometric techniques, emphasizing the need to use chemometric models in the poultry meat industry, as they consider all possible relationships among the variables. Further studies should be carried out to determine the relationship between biogenic amines and other bacterial groups, as well as to establish the biochemical parameters of the poultry meat species to better understand of this relationship. In addition, analyses of the biogenic amine behavior before the point of sale, such as during slaughter and processing, are also welcome.

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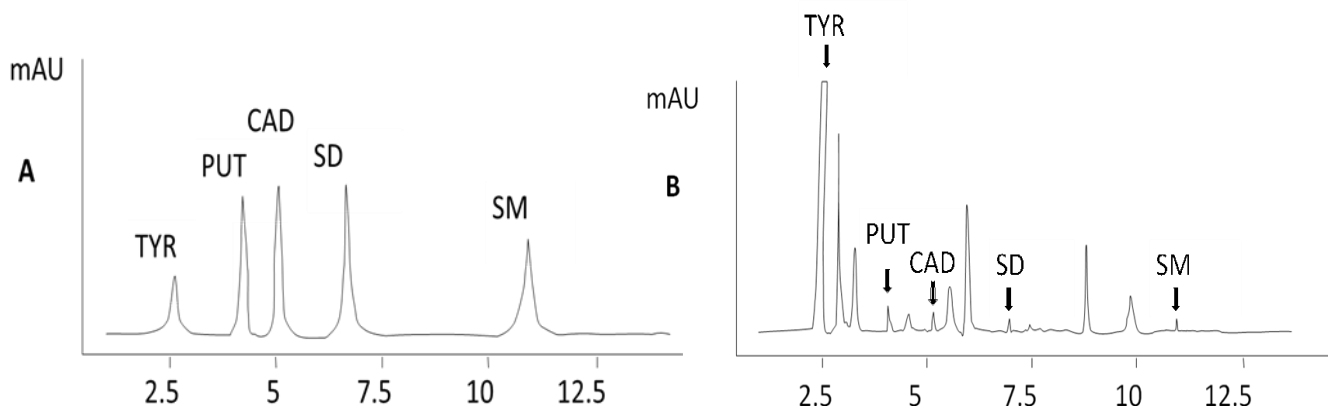
Figure captions

Fig. 1. Chromatograms of mix amines standard solution (A) and chicken breast meat sample (B). Abbreviations: TYR = Tyramine, PUT = Putrescine, CAD = Cadaverine, SD = Spermidine and SM = Spermine.

Fig. 2a. Plot of factor loading and observations scores obtained by the principal component analyses (PCA) performed on different poultry meats. (QUA = quail, DUC = duck, ORG = Organic chicken, CONV= Conventional chicken, FREE = Free-range chicken, Tyr = Tyramine, Put = Putrescine, Cad = Cadaverine, Sp = Spermidine, Sm = Spermine, Ent = Enterobacteriaceae, Mes = Total aerobic mesophilic bacteria)

Fig. 2b. Dendrogram of the hierarchical cluster analysis (HCA) of samples assessed by the microbiological and biogenic amines parameters. (QUA = quail, DUC = duck, ORG = Organic chicken, CONV= Conventional chicken, FREE = Free-range chicken)



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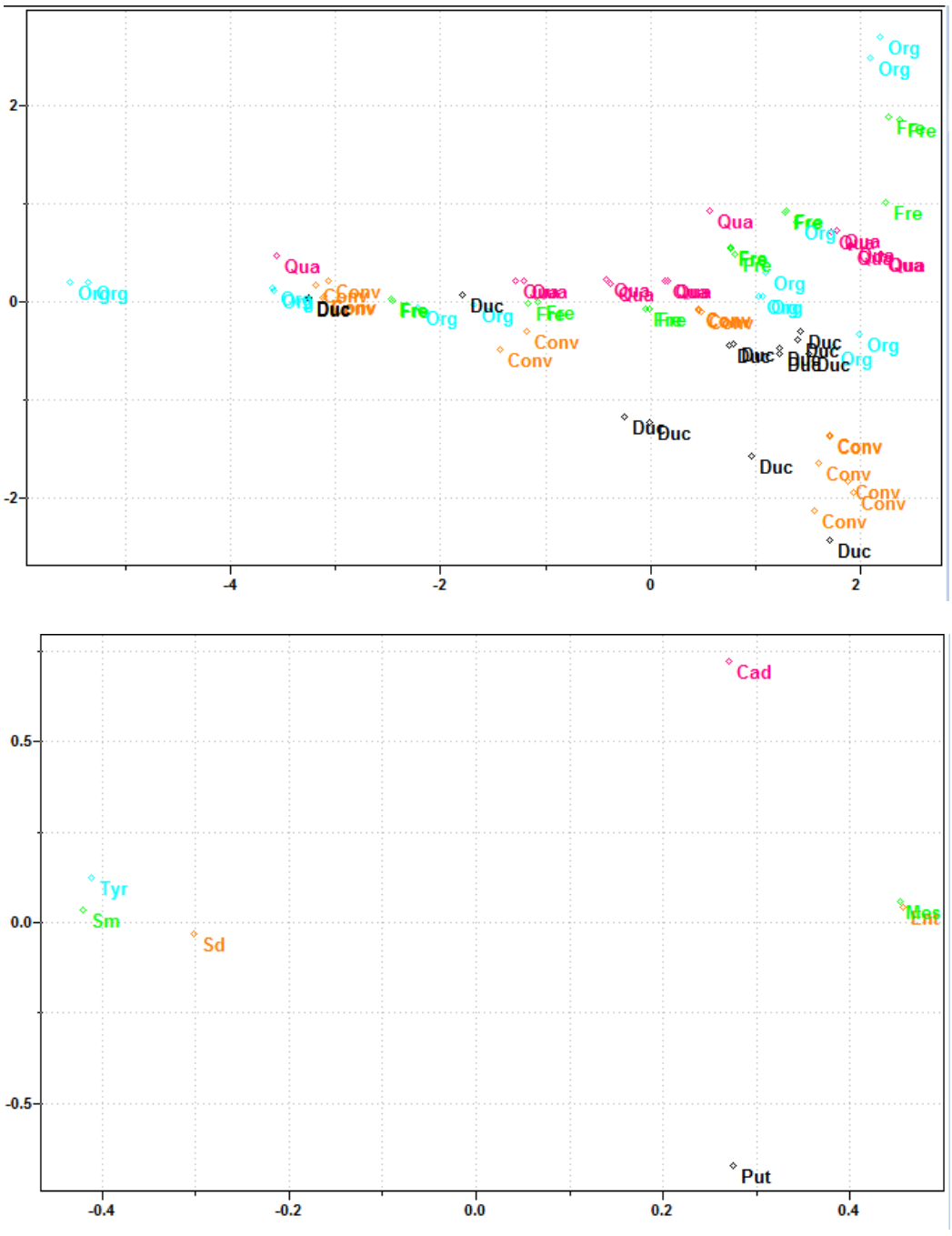

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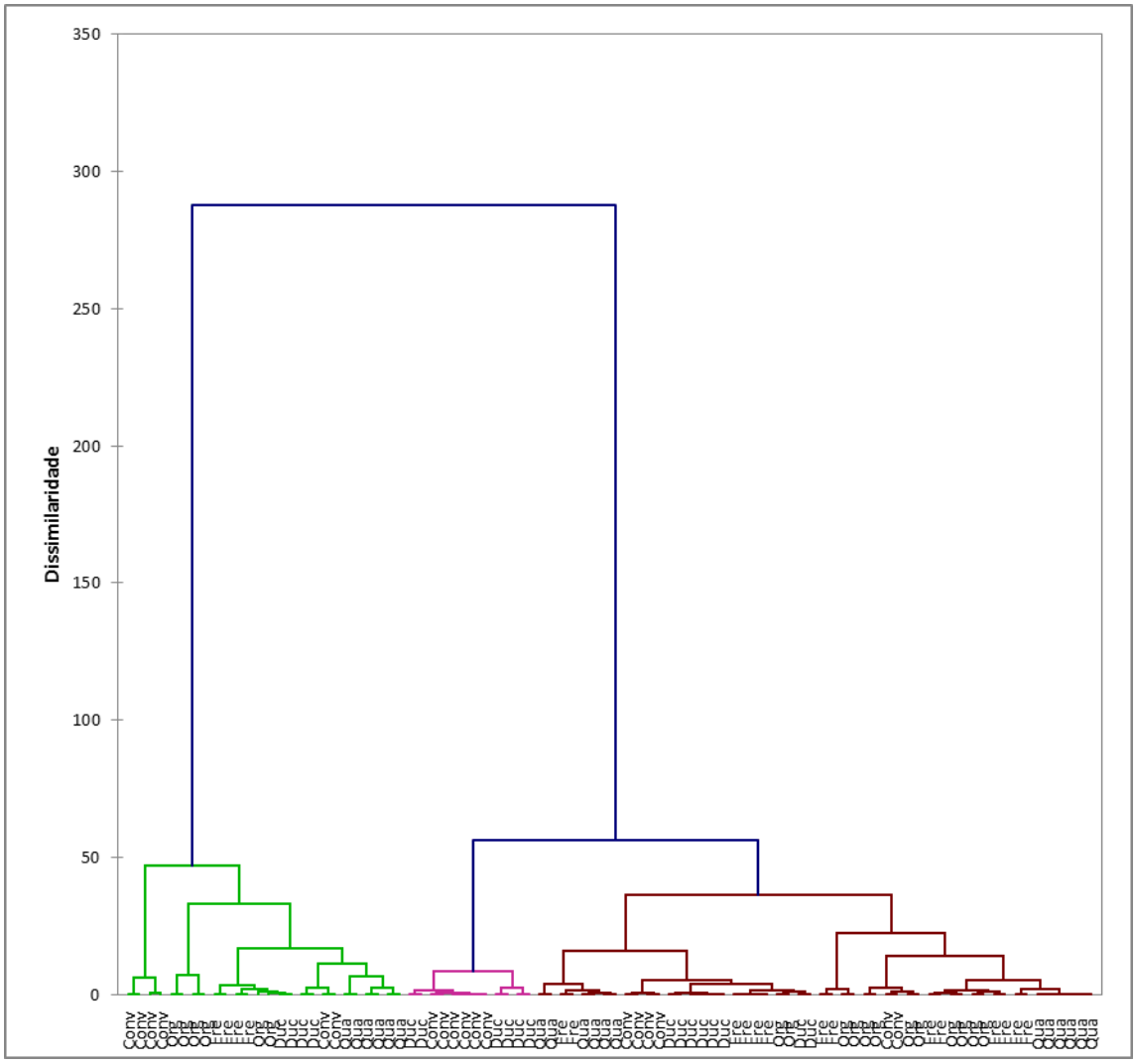

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Table captions

Table 1a. Levels of tyramine, putrescine and cadaverine (mg kg^{-1}) in chicken (conventional, free-range and organic), duck and quail meats stored at 4±1°C for 17 days.

Table 1b. Levels of spermine and spermidine (mg kg^{-1}) in chicken (conventional, free-range and organic), duck and quail meats stored at 4±1°C for 17 days.

Table 2. Bacterial parameters of chicken (conventional, free-range and organic), duck and quail meats stored at 4±1°C for 17 days.

Table 3. Results of Pearson correlation between biogenic amines and bacterial groups in different poultry meat tested.

Table 4. Classification results for the supervised patterns

Table 1a. Levels of tyramine, putrescine and cadaverine (mg kg^{-1}) in chicken (conventional, free-range and organic), duck and quail meats stored at 4±1°C for 17 days.

	Storage (days)								
Parameters	**1**	**3**	**5**	**7**	**9**	**11**	**13**	**15**	**17**
TYR									
Conventional	221.6 ± 2.8 **a2**	198.3 ± 1.3 **b2**	112.8 ± 6.1 **d2**	88.8 ± 2.6 **e2**	124.0 ± 1.3 **c1**	46.7 ± 0.5 **f2**	24.2 ± 0.1 **g2**	20.1 ± 1.9 **g2**	3.6 ± 0.2 **h23**
Free-range	96.6 ± 4.0 **a3**	56.2 ± 0.1 **cd4**	52.6 ± 3.14 **c3**	67.7 ± 0.4 **b3**	60.9 ± 1.0 **bd3**	64.4 ± 0.1 **b12**	38.8 ± 0.6 **e1**	34.9 ± 1.5 **e1**	4.6 ± 0.3 **f2**
Organic	214.9 ± 4.3 **a2**	196.9 ± 0.7 **b2**	134.4 ± 2.9 **c2**	97.2 ± 1.2 **d12**	91.6 ± 1.0 **d2**	80.3 ± 2.7 **e1**	30.7 ± 4.2 **f12**	14.0 ± 0.8 **g3**	7.9 ± 1.2 **h1**
Duck	135.5 ± 2.3 **a4**	92.1 ± 5.5 **b3**	49.9 ± 2.0 **c3**	27.0 ± 2.2 **d4**	13.0 ± 1.0 **e4**	8.5 ± 0.2 **f3**	4.4 ± 0.1 **ef3**	3.9 ± 0.1 **fg4**	2.4 ± 0.4 **g34**
Quail	356.8 ± 19.4 **a1**	272.3 ± 17.8 **b1**	177.9 ± 10.3 **c1**	106.7 ± 4.1 **d1**	93.5 ± 4.4 **d2**	50.6 ± 9.9 **e2**	28.2 ± 2.0 **f2**	20.6 ± 1.4 **f2**	1.7 ± 0.0 **g4**
PUT									
Conventional	1.2 ± 0.1 **d1**	1.5 ± 0.1 **d1**	8.7 ± 2.4 **c1**	6.2 ± 0.3 **c1**	7.1 ± 0.1 **c1**	30.2 ± 0.1 **b1**	45.9 ± 1.8 **a1**	41.1 ± 2.5 **a1**	45.2 ± 1.4 **a1**
Free-range	ND	0.4 ± 0.1 **e2**	6.7 ± 0.3 **c1**	14.0 ± 0.8 **b2**	17.2 ± 0.8 **ab2**	20.2 ± 2.1 **a2**	9.9 ± 0.4 **c2**	2.1 ± 0.0 **d2**	ND
Organic	ND	3.0 ± 0.2 **b3**	5.6 ± 0.0 **c1**	17.3 ± 1.6 **b2**	28.7 ± 3.5 **a3**	7.2 ± 1.1 **c3**	5.5 ± 0.5 **c3**	4.2 ± 0.6 **d23**	ND
Duck	0.2 ± 0.0 **f2**	0.4 ± 0.0 **f2**	31.0 ± 0.7 **b2**	34.7 ± 3.6 **b3**	54.8 ± 2.3 **a4**	18.2 ± 0.9 **c4**	13.2 ± 1.1 **d4**	12.7 ± 0.7 **d3**	8.2 ± 0.1 **e2**
Quail	ND	2.4 ± 0.4 **g4**	5.8 ± 0.2 **f1**	9.2 ± 0.2 **e1**	13.9 ± 0.1 **c12**	11.9 ± 0.1 **d4**	15.9 ± 0.3 **b4**	17.0 ± 0.1 **a4**	17.7 ± 0.2 **a3**
CAD									
Conventional	ND	0.1 ± 0.0 **e1**	0.1 ± 0.0 **e1**	0.2 ± 0.0 **de1**	0.2 ± 0.1 **de1**	0.5 ± 0.1 **d1**	1.8 ± 0.1 **c1**	4.0 ± 0.2 **b1**	5.7 ± 0.1 **a1**
Free-range	ND	ND	2.9 ± 0.1 **f2**	11.4 ± 0.7 **d2**	22.8 ± 2.7 **b2**	33.5 ± 1.2 **a2**	16.3 ± 0.4 **c2**	7.0 ± 0.1 **e2**	5.5 ± 0.1 **e1**
Organic	0.1 ± 0.0 **f1**	0.3 ± 0.1 **f2**	1.5 ± 0.1 **e3**	3.7 ± 0.2 **d3**	7.6 ± 0.2 **c34**	6.1 ± 0.0 **c3**	11.6 ± 0.8 **b3**	33.2 ± 2.1 **a3**	ND
Duck	ND	ND	4.4 ± 0.2 **a4**	4.3 ± 0.3 **a4**	4.2 ± 0.1 **a3**	3.8 ± 0.2 **ab4**	3.3 ± 0.1 **b1**	1.3 ± 0.1 **c4**	ND
Quail	ND	ND	3.8 ± 0.2 **e5**	7.0 ± 0.1 **d4**	10.1 ± 0.1 **c4**	19.6 ± 1.3 **a5**	17.7 ± 0.3 **ab2**	16.6 ± 0.1 **b5**	16.3 ± 0.1 **b2**

Abbreviations: TYR = tyramine, PUT = putrescine, CAD = cadaverine, ND = not detectable.
Values represent the mean of three measurements (n = 3).
Different letters in the same row and different numbers in the same column represent significant differences ($p < 0.05$).

Table 1b. Levels of spermine and spermidine (mg kg^{-1}) in chicken (conventional, free-range and organic), duck and quail meats stored at 4±1°C for 17 days.

	Storage (Days)								
Parameters	**1**	**3**	**5**	**7**	**9**	**11**	**13**	**15**	**17**
SPD									
Conventional	7.8 ± 0.3 **b1**	9.7 ± 0.1 **a1**	8.4 ± 0.5 **b1**	6.4 ± 0.0 **c2**	6.3 ± 0.0 **c3**	6.3 ± 0.0 **c2**	6.3 ± 0.0 **c2**	6.3 ± 0.0 **c3**	6.3 ± 0.0 **c2**
Free-range	6.7 ± 0.0 **ab2**	6.8 ± 0.2 **a2**	6.5 ± 0.1 **bc2**	6.4 ± 0.0 **c2**	6.3 ± 0.0 **c3**	6.3 ± 0.0 **c2**	6.3 ± 0.0 **c12**	6.4 ± 0.0 **c2**	6.3 ± 0.0 **c2**
Organic	7.2 ± 0.2 **a12**	6.3 ± 0.0 **b3**	6.5 ± 0.6 **b1**	6.3 ± 0.1 **b2**	6.4 ± 0.0 **b2**	6.4 ± 0.0 **b1**	6.4 ± 0.0 **b1**	6.4 ± 0.0 **b1**	6.3 ± 0.1 **b2**
Duck	7.7 ± 0.1 **a1**	6.4 ± 0.1 **b3**	6.8 ± 0.3 **b1**	7.2 ± 0.2 **a1**	6.3 ± 0.0 **b3**	6.3 ± 0.0 **b2**	6.4 ± 0.1 **b1**	6.3 ± 0.0 **b2**	6.6 ± 0.1 **b1**
Quail	7.1 ± 0.1 **a12**	6.8 ± 0.1 **b2**	6.7 ± 0.0 **b2**	6.6 ± 0.0 **b2**	6.6 ± 0.0 **b1**	6.5 ± 0.0 **b1**	6.3 ± 0.0 **b12**	6.3 ± 0.0 **b3**	6.3 ± 0.0 **b2**
SPM									
Conventional	9.9 ± 0.3 **a23**	5.1 ± 0.2 **b3**	1.9 ± 0.0 **c2**	1.7 ± 0.1 **c12**	1.5 ± 0.0 **c3**	1.5 ± 0.0 **c1**	1.5 ± 0.0 **c1**	1.5 ± 0.0 **c1**	ND
Free-range	8.9 ± 0.1 **a2**	5.5 ± 0.2 **b3**	1.7 ± 0.1 **c2**	1.7 ± 0.0 **c12**	1.6 ± 0.0 **c2**	1.5 ± 0.0 **c1**	1.5 ± 0.0 **c1**	1.5 ± 0.0 **c1**	1.5 ± 0.0 **c1**
Organic	23.7 ± 0.1 **a1**	17.5 ± 0.3 **b1**	8.2 ± 1.0 **c1**	1.6 ± 0.0 **d2**	1.5 ± 0.0 **d3**	1.5 ± 0.0 **d1**	1.5 ± 0.0 **d1**	1.5 ± 0.0 **d1**	ND
Duck	10.4 ± 0.2 **a3**	9.8 ± 0.2 **a2**	7.5 ± 0.1 **b1**	1.8 ± 0.0 **c1**	1.5 ± 0.0 **c3**	1.5 ± 0.0 **c1**	1.5 ± 0.0 **c1**	1.5 ± 0.0 **c1**	ND
Quail	9.4 ± 0.5 **a23**	5.1 ± 0.2 **b3**	2.2 ± 0.0 **c2**	1.8 ± 0.1 **c1**	1.8 ± 0.0 **c1**	1.5 ± 0.0 **c1**	1.5 ± 0.0 **c1**	1.5 ± 0.0 **c1**	ND

Abbreviations: SPD = spermidine, SPM = spermine, ND = not detectable.
Values represent the mean of three measurements (n = 3).
Different letters in the same row and different numbers in the same column represent significant differences ($p < 0.05$).

Table 2. Bacterial parameters of chicken (conventional, free-range and organic), duck and quail meats stored at 4±1°C for 17 days.

Parameters	Storage time (days)								
	1	**3**	**5**	**7**	**9**	**11**	**13**	**15**	**17**
TAMB									
Conventional	3.93 ± 0.01 **h1**	4.20 ± 0.01 **g1**	4.46 ± 0.01 **f3**	6.09 ± 0.02 **e3**	6.74 ± 0.04 **b2**	7.48 ± 0.01 **a1**	6.28 ± 0.02 **d4**	6.46 ± 0.04 **c4**	6.07 ± 0.02 **e3**
Free-range	3.52 ± 0.01 **i2**	4.04 ± 0.03 **h2**	4.64 ± 0.01 **g2**	6.34 ± 0.02 **d2**	7.75 ± 0.01 **a1**	6.74 ± 0.01 **b3**	6.59 ± 0.02 **c3**	6.18 ± 0.01 **e5**	6.00 ± 0.01 **f4**
Organic	ND	3.04 ± 0.01 **e5**	3.08 ± 0.01 **e5**	8.71 ± 0.01 **a1**	7.71 ± 0.01 **b1**	7.20 ± 0.01 **c2**	7.18 ± 0.01 **c1**	7.20 ± 0.01 **c1**	6.72 ± 0.01 **d2**
Duck	3.18 ± 0.01 **g4**	3.72 ± 0.01 **f3**	4.81 ± 0.01 **e1**	5.60 ± 0.01 **c4**	5.11 ± 0.03 **d3**	7.43 ± 0.03 **a1**	7.14 ± 0.03 **b1**	6.57 ± 0.02 **c3**	5.51 ± 0.03 **c5**
Quail	3.40 ± 0.02 **c3**	3.56 ± 0.01 **c4**	4.03 ± 0.03 **bc4**	4.50 ± 0.01 **bc5**	4.98 ± 0.01 **b4**	4.79 ± 0.01 **b4**	6.85± 0.02 **a2**	6.86 ± 0.01 **a2**	6.95 ± 0.01 **a1**
Enterobacteriaceae									
Conventional	2.00 ± 0.01 **h2**	3.00 ± 0.02 **g2**	4.49 ± 0.03 **f2**	6.59 ± 0.01 **b2**	6.18 ± 0.01 **c3**	6.74 ± 0.01 **a2**	5.23 ± 0.01 **e4**	5.36 ± 0.02 **d5**	5.38 ± 0.02 **d4**
Free-range	ND	3.60 ± 0.04 **f1**	4.76 ± 0.01 **e1**	6.58 ± 0.01 **c2**	7.75 ± 0.01 **a1**	7.23 ± 0.02 **b1**	5.48 ± 0.01 **d3**	5.53 ± 0.01 **d4**	5.39 ± 0.01 **d4**
Organic	ND	ND	2.48 ± 0.01 **g5**	8.72 ± 0.01 **a1**	7.36 ± 0.01 **b2**	6.72 ± 0.01 **c2**	6.26 ± 0.01 **e1**	6.40 ± 0.01 **d2**	6.16 ± 0.01 **f2**
Duck	ND	2.30 ± 0.01 **f4**	4.18 ± 0.01 **e3**	5.48 ± 0.01 **d3**	5.85 ± 0.01 **c4**	5.57 ± 0.01 **d3**	6.15 ± 0.01 **b2**	6.20 ± 0.01 **a3**	5.88 ± 0.01 **c3**
Quail	2.48 ± 0.01 **i1**	2.84 ± 0.03 **h3**	3.42 ± 0.02 **g4**	4.01 ± 0.02 **f4**	4.60 ± 0.02 **e5**	4.15 ± 0.03 **d4**	6.23 ± 0.02 **c1**	6.78 ± 0.02 **b1**	6.89 ± 0.01 **a1**

Values represent the mean of three determinations (n=3)
Different letters in the same row and different numbers in the same column represent significant differences ($p < 0.05$).
ND = not detected
TAMB = Total aerobic mesophilic bacteria

Table 3. Results of Pearson correlation between biogenic amines and bacterial groups in different poultry meat tested.

	Tyramine	Putrescine	Cadaverine	Spermine	Spermidine
Conventional					
TAMB	-0.758*	0.592	0.316	-0.726*	-0.853*
ENT	-0.728*	0.412	0.193	-0.848*	-0.755*
Organic					
TAMB	-0.799*	0.535	0.465	-0.936**	-0.780*
ENT	-0.790*	0.561	0.437	-0.927**	-0.606
Free-range					
TAMB	-0.346	0.726*	0.795*	-0.800*	-0.892*
ENT	-0.398	0.743*	0.737*	-0.905**	-0.787*
Duck					
TAMB	-0.864*	0.070	0.473	-0.817*	-0.577
ENT	-0.993**	0.407	0.510	-0.928**	-0.650
Quail					
TAMB	-0.866*	0.934**	0.837*	-0.700*	-0.918**
ENT	-0.878*	0.945**	0.820*	-0.730*	-0.926**

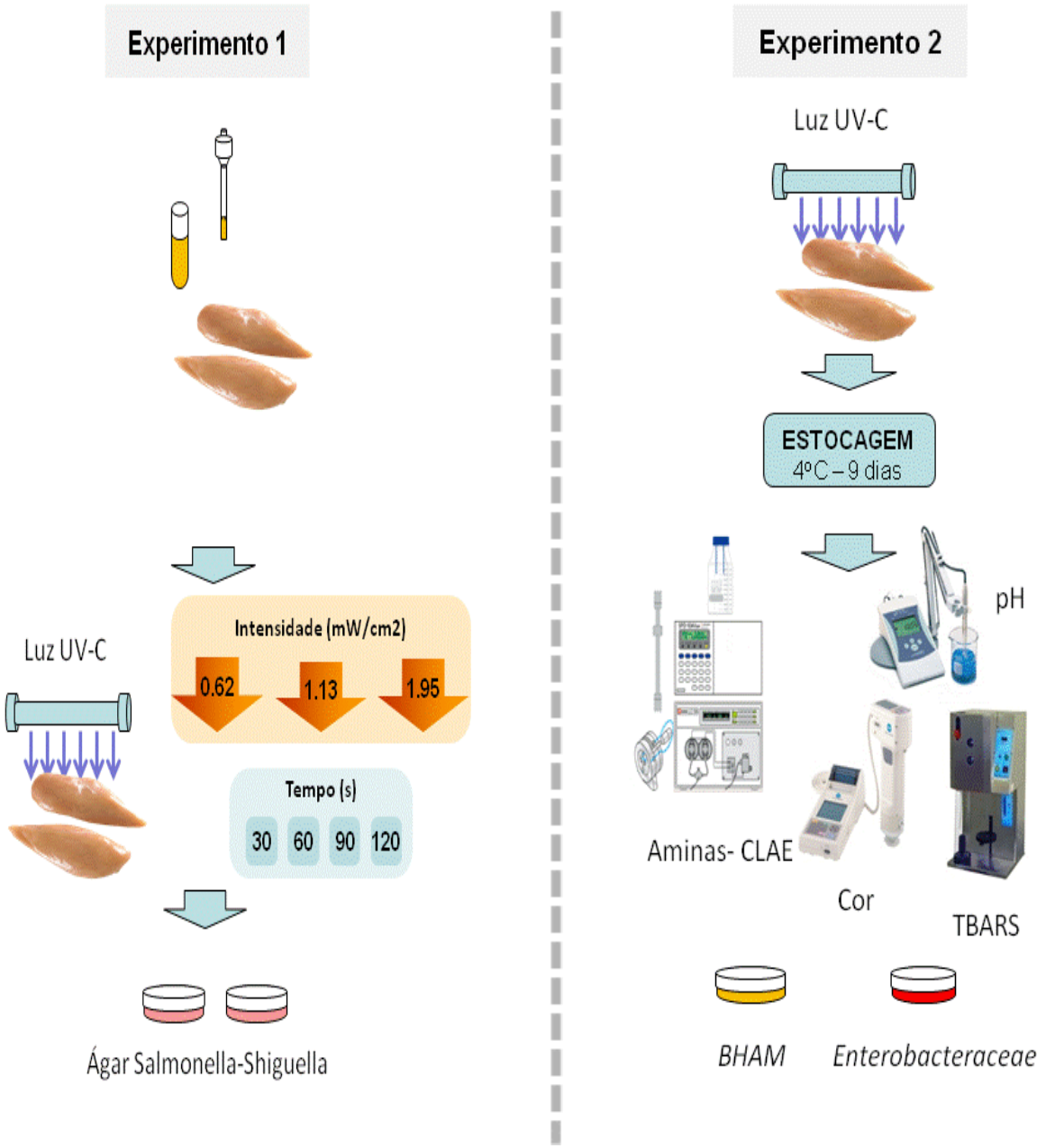
Values represent the mean of three determinations (n=3)
Abbreviation: TAMB = Total aerobic mesophilic bacteria, ENT = Enterobacteriaceae
** $p < 0.001$, *$p < 0.05$

Table 4. Classification results for the supervised patterns

Technique	Class	Recognition Ability (%)	Prediction Ability (%)
KNN (K=2); inverse squared Euclidean distance	CONV	100	100
	FREE	100	100
	ORG	100	100
	DUC	100	100
	QUA	100	100
SIMCA ; normal range, α=0.05	CONV	100	100
	FREE	100	50
	ORG	100	100
	DUC	100	100
	QUA	100	100

Abbreviations: QUA = quail, DUC = duck, ORG = Organic chicken, CONV= Conventional chicken, FREE = Free-range chicken

3.4 ARTIGO 4: EFFECT OF UV-C RADIATION ON THE QUALITY OF CHICKEN MEAT DURING STORAGE (Será enviado para publicacão)



EFFECT OF UV-C RADIATION ON THE QUALITY OF CHICKEN MEAT DURING STORAGE

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ABSTRACT

The efficiency of low (0.62 mW/cm²), medium (1.13 mW/cm²) and high 1.95 mW/cm²) UV-C intensities at different exposition times in reduction of *Salmonella* species inoculated in twenty four chicken breast and the quality changes (total viable count, Enterobacteriaceae, TBARS, pH, color and biogenic amines) in forty eight chicken breast meats irradiated with the same intensities in 90 seconds and storage at 4°C for 9 days were tested. The best efficiency in bacterial reduction (between 0.33 and 0.60 Log CUF/g) was observed in 90 and 120 seconds in all UV-C intensities. In the storage time the UV-C irradiation showed a reduction in the initial load and retarded the bacterial growth curve of TAMB and *Enterobacetriaceae* with high and medium intensities; increased the biogenic amines values, suggesting that this parameter is not suitable like a quality indicator in these meats and without significant variation in yellowness (b* values) On the other hand, no significant changes were observed in pH, TBARS, lightness (L*) and redness (a*) color values The UV-C light was efficient in reduction of *Salmonella* species and it can be used as an alternative technology to improve the bacteriological quality of packing chicken meat at the end of line process without significant changes in the physicochemical parameters.

Keywords: bacterial, biogenic amines, color, pH, TBARS, ultraviolet.

INTRODUCTION

Although chicken meat is normally cleansed in chilled water before packaging, microbial cross-contamination is inevitable and the microbiological control standards in this products may include the reduction of external contamination with spoilage and pathogenic microorganisms. In the poultry industry, bacteria adhere principally in surfaces (skin, carcass cavity, meat deboned, etc) representing the main sources of contamination of the underlying tissues during portioning, skinning and boning. There is a increased in contamination problems since chicken carcasses follow a manual or technological processed to be divided into pieces to satisfied the preferences of consumers (Kondjoyan and Portanguen, 2008).

These facts, couple with the consumer demand for safe food, have renewed the interest in process to help with the decontamination in surface of food products. Various processing methods have been proposed as potential decontamination interventions, alone or in combination, include gamma irradiation, continuous and pulsed ultraviolet (UV) light, high hydrostatic pressure, infrared technology, electro-magnetic fields, sonication, microwaves, etc. Among these, UV light irradiation is the most suitable and promising technology which involves discharge and translocation of energy in the form of waves or particles through space or a food without inducing radioactivity. The process destroy injures microorganisms directly by damaging bacterial DNA or indirectly by free radicals formed during water radiolysis (Byelashov and Sofos, 2009). These non-thermal technologies can deliver food products without hazardous microorganisms and enzymes that may reduce the nutritional and sensory characteristics of foods, which are often changed when thermal processes are applied (Guerrero-Beltr·n and Barbosa-C·novas, 2004, Lado and Yousef, 2002)

The beneficial effect of UV-C irradiation had been tested in chicken meat. Wallner-Pendleton et al. (1994) suggested that UV radiation can reduce *Salmonella* surface contamination without negatively affecting carcass color or increasing rancidity of the meat. Kim et al. (2002) showed a reduction ranges from 0.36 to 1.28 log cycles after UV treatment for 3 min at 500 mW/cm2 in all selected pathogens (*Salmonella* Thipymiurium, *Listeria monocytogenes* and *Escherichia coli)*. Lyon et al. (2007) showed that this process could be used to reduce the negative effect of raw poultry as a transmission vector of *Listeria monocytogenes* into a poultry processing plant without negatively affecting meat color

Although these authors showed no alteration in parameters like color and oxidative rancidity, there is a lack of information of changes in other biochemical indicator of meat quality.

Biogenic amines are low molecular weight substances, formed mainly by decarboxylation of specific amino acids present in food through the action of bacterial enzymes during storage (Silla Santos, 1996); due to this fact, the total amount of the different amines formed strongly depends on the nature of the food and the microorganisms present. Therefore, biogenic amines can be used as an indicator of microbial development (Vinci and Antonelli, 2002). The objective of this study was determined the effect of UV-C irradiation on *Salmonella* serotypes inoculated on chicken breast as well as the effect of some bacteriological (TAMB and Enterobacteriaceae) and physicochemical (biogenic amines, pH, TBARS and color) parameters on packing chicken breasts storage at 4±2 °C during 9 days.

MATERIALS AND METHODS

UV-C equipment

In order to perform the experiments, a stainless steel barrel-shaped chamber was constructed (Figure 1). Twelve UV-C lamps (Six of 30W and six of 55W; OSRAM HNS, OFR) were mounted and distributed in interspersed positions inside the chamber. Nylon net was used to put the samples between both sides of chamber. Two switches were installed to control each group of lamps independently. The UV-C lamps were warmed up for 30 min before irradiation. The intensity was varied depending on each group of lamps: 30 W (0.62 mW/cm²), 55 W (1.13 mW/cm²) and 30 + 55W (1.95 mW/cm²), these values were determined using an UV radiometer (MRUR-203, Instrutherm LTDA., São Paulo, Brazil). Different locations throughout the nylon net were tested by UV radiometer in order to determinate the highest irradiance inside the chamber.

Evaluation of UV-C in chicken meat inoculated with *Salmonella* species

Meat samples. Twenty four chicken breasts (m. *Pectoralis profundus*) were purchased from a local market in Niteroi city, Rio de Janeiro (Brazil). Each breast was aseptically placed on polyethylene film pack and stored at 4±2 °C and used for the experiment within 24 h.

Preparation of inoculums. A total of five serotypes of *Salmonella enterica*: Enteritidis (ATCC 13076), Typhi (ATCC 19214), Thypimurium (ATCC 14028), Gallinarum (ATCC9184) and Arizonae (ATCC13314) were used in this study. All serotypes were obtained from the Fiocruz Institute (Rio de Janeiro, Brazil). Cultures were stored at -70 °C in brain heart infusion (BHI) broth with 20% of glycerol until the beginning of the experiment.

Prior to use, each culture were grown independently in Falcon tubes containing 12 mL of BHI broth at 37 °C for 24 h, this process was repeated twice. The incubation for 24 h allowed the respective bacteria to approach the stationary phase of growth at a concentration of approximately Log 8 CFU/ml. After the last incubation, cultures were centrifuged at 1000 *g* for 15 min at 4°C. Cell pellets washed three times with sterile phosphate-buffered saline (PBS, pH 6.0). Finally, equal aliquots of each strain were combined into a sterile Falcon tube in order to obtain an inoculum solution cocktail of five serotypes. The bacterial concentrations were determined by measuring optical density at 600nm (OD600) by UV spectrophotometer (Smartspec Plus, BioRad, Hercules, CA, USA).

Inoculation of chicken meat. One milliliter of inoculum solution with OD600 corresponding to approximately 5.0 x 10^8 cells was spotted onto the surface of the chicken breast. The samples were allowed to dry for 15 min before exposure to UV.

UV-C irradiation. The inoculated chicken breasts were exposed to three UV-C intensities (0.62, 1.13 and 1.95 mW/cm²) in four times (30, 60, 90 and 120 seconds). The irradiation was performed in a dark room to minimize photo reactivation of the pathogenic bacteria. The chicken breasts on polystyrene film pack were placed in a central area (10 x 40 cm²) of the nylon net, previously determinate with a best place with a constant and high UV-C irradiation. The samples were located at a distance of 14 cm of light sources and irradiated on both surfaces. After irradiation of samples, bacteriological tests were performed.

Bacteriological analysis. After the UV-C irradiation, 25 g of chicken meat were removed using a sterile scalpel and mixed with 225 mL of peptone water (0.1% sterile peptone, w/v) in a sterile bag and homogenized using a stomacher (Stomacher 80, Seward, London, UK) for 2 min. An 8-fold dilution series were prepared in peptone water and 0.1 mL of each dilution was spread plated in duplicate on Salmonella-Shiguella agar. The plates were incubated at 37 °C for 48 h. One additional breast was tested for the initial presence of *Salmonella* species (negative control). Mean counts were calculated and converted to log $CFU.g^{-1}$.

Effect of UV-C in chicken meat quality during storage time at 4±2 °C

Meat Samples and UV-C irradiation. Forty eight chicken breasts were purchased in the same way described above. Each chicken breast was aseptically placed on film pack (Polyvinyl chloride, thickness 0.16 µ, previously tested to allow passage of the radiation) and the total was equally divided Control (Without UV-C irradiation) and three UV-C treatments

irradiated with different intensities for 90 seconds: Low intensity (0.62 mW/cm²), Medium intensity (1.13 mW/cm²) and High intensity (1.95 mW/cm²). After that, all samples were stored at 4±2°C for 9 days.

Reagents and standards. All reagents used in the present study were of analytical grade. Biogenic amines standard were purchased from Sigma Aldrich®. Stock solution with 40 µg of cadaverine, putrescine, tyramine, spermidine and spermine were prepared in 0.1N HCl and stored at 4±1°C. Different dilutions were performed and used for calibration curve constriction, limits of detection and quantification and recovery of the proposed method.

***pH* measurement.** The pH values were performed by using a digital pH meter (Digimed® DM-22) equipped with a DME-R12 electrode (Digimed®) after homogenization of each 10 g of muscle sample in 90 mL of distilled water (Conte-Júnior et al., 2008)

Measurement of lipid oxidation. The degree of lipid oxidation of the chicken breasts was determined using the method of 2-thiobarbituric acid reactive substances (TBARS) according to Tarladgis et al. (1960). Thiobarbituric acid reactive substances (TBARS) values were calculated from a standard curve and expressed as mg malonaldehyde per kg sample (MDA/kg).

Instrumental color measurement. Colors of the samples were analyzed using a colorimeter CR- 400 Chroma Meter (Minolta, Osaka, Japan) previously calibrated. Color was read at 2 different locations with two replication and expressed using the system of lightness (**L***), redness (**a***), and yellowness (**b***).

Bacteriological analyses. A 25 g aliquot of each sample was transferred aseptically to sterile homogenization bags with 225 ml 0.1% peptone water, and serial dilutions (10^{-2} to 10^{-5}) were prepared (Álvarez-Astorga et al. 2002). The total aerobic mesophilic bacteria (TAMB) were enumerated on Plate Count Agar (PCA) by the pour-plating method, followed by incubation at 35°C for 48 h (APHA, 1998). For the Enterobacteriaceae count, serial dilutions were pour-plated in Violet Red Bile Glucose Agar (VRBGA) with an overlayer and incubated at 35°C for 48 h. After the incubation period, the bacterial colonies were counted using a Quebec colony counter (Leica, Inc., Buffalo, USA) (Leite & Franco, 2009). The total count results were transformed into Log CFU g^{-1}.

Biogenic amine determination. The extraction and derivatization procedures were carried out according to Lázaro et al. (2013). In brief: Chicken meat was extracted with perchloryc

acid 5%, neutralized with NaOH (pH > 12) and derivatized with addition of benzoyl chloride (40 μL); then, the mixture was extracted with diethyl ether and evaporated to dryness under a stream of nitrogen. Finally, the residue was dissolved in 1000 μL of mobile phase (acetonitrile:water) and stored at 4 ± 1 °C.

The chromatographic system consisted of a Shimadzu Prominence UFLC apparatus (Shimadzu, Kyoto, Japan) equipped with a SPD-M20A diode array detector and a LC-20AD quaternary pump, SIL-20AC auto sampler, CTO-20A column oven, DGU-20A5 degasser and a CBM-20A communication bus module. Amines separation were performed on C18 Spherisorb ODS2 (15 x 0.46 cm id., 5 μm, Waters) column equipped with a Supelco, Ascentis C18 (2 x 0.40 cm, id. 5 μm) guard column, in isocratic condition. The mobile phase consisted of acetonitrile (Tedia®) and ultrapure water purified on Millipore Simplicity (Millipore, Molsheim, France), 42:58 (v/v), previously degassed in ultrasonic bath (Cleaner USC 2800 A). The chromatography conditions were: flow rate of 1 mL min^{-1}, injection volume of 20 μL, column temperature of 20°C. Benzoylated polyamines were detected by UV absorption at 198 nm, each sample was injected two times and total run time of 15 minutes. Injection of pure acetonitrile for 10 minutes was used between each sample for cleaning the HPLC system. Presence of biogenic amines were identified by retention time and quantified by peak area.

Statistical Analysis. Trials were performed in duplicate at each sampling. Bacterial counts were converted to log10 CFU/g of chicken meat. The statistic study of first experiment was carried out with analysis of variance (ANOVA); the means were compared with Tukey test ($p \leq 0.05$). The analyses were performed on GraphPad Prism® (Ver. 5.00., 2007) package for windows (GraphPad, 2007).

RESULTS AND DISCUSSION

UV-C in chicken meat inoculated with *Salmonella* species

Our results showed that the bacterial Log reduction increasing with high UV-C intensities and prolonged the time of exposition (Table 1). The best efficiency for was observed in 90 and 120 seconds of expositions in low, medium and high intensities used (0.62, 1.13 and 1.95 $mW.cm^{-2}$). In this conditions can be observed a Log reduction between 0.33-0.60.

These results are consistent with others researches focus the UV-C irradiation in chicken meat; Wallner-Pendleton et al. (1994) evaluated the potential of 82.5 $mW.cm^{-2}$ for 60 seconds

in broiler carcasses inoculated with *Salmonella* Typhimurium and found a reduction of 0.5 log. Sommers et al. (2010) showed that doses of 2–4 J/cm2 inactivated between 0.4 and 0.6 log of *Salmonella* spp. inoculated onto the surfaces of boneless skinless chicken breasts and chicken drumsticks. Haughton et al. (2011) studied the susceptibility of different bacterium inoculated in chicken meat using an intensity of 6 $mW.cm^{-2}$ in 8, 16 and 32 seconds; as expected, the maximum time showed maximum reductions of 0.76, 0.98, 1.34, 1.76, and 1.29 log CFU/g were achieved for *C. jejuni, E. coli, Salmonella* Enteritidis, total viable counts (TVC).

The benefic effect of UV-C radiation in load reduction had been reported in others microorganisms, Kim et al. (2002) used a intensity of 0.5 $mW.cm^{-2}$ in 180 seconds on inoculated chicken meat demonstrated a reduction of 0.46, 0.36, and 0.93 log for *Listeria monocytogenes*, *Salmonella* Typhimurium and *E. coli* O157: H7, respectively. Lyon et al. (2007) reported an approximate 2 Log reduction in viable *Listeria monocytogenes* after irradiation of 1000 μW/cm2 for 5 min on breast chicken fillets. Finally, Isohanni and Lyhs (2009) evaluated the effect of 39.2 $mW.cm^{-2}$ for 60 seconds on broiler meat surfaces inoculated with *C. jejuni* achieved a reduction of 0.7 log.

It is interesting to note that bacterial reduction after the UV-C irradiation was low in all researches with chicken meat. Wallner-Pendleton et al. (1994) determinate this trend is due to the chicken carcasses do not have smooth surfaces, and the skin is lined irregularly with numerous feather follicles. Fresh food products can be processed using UV light as a germicidal medium to reduce the food-borne microbial load. However, it does not penetrate the target very deeply (Guerrero-Beltr·n and Barbosa-C·novas, 2004).

UV-C in chicken meat quality during storage time at 4±2 °C

After the UV-C evaluation in inoculated chicken meat, two exposition times (90 and 120 seconds) were more effectives in log reduction. For determinate the effect of UV-C in chicken meat quality it was chosen 90 seconds as exposition time because it was the minor time to reach an optimal bacterial reduction .

Bacteriological count

Only the high UV-C intensity showed a significant effect ($p<0.05$) of the mesophilic and Enterobacteriaceae growth. In the storage, all groups followed a constant increment but in the 8^{th} day the control, low and medium intensity groups become to decrease while the high

intensity group continues to grow (Figure 2). This behavior suggested that the high and medium UV-C intensities slightly reduced the initial bacteria load, probably only in the surface, but this reduction was sufficient to retard the bacterial growth curve in this groups. It is known that a count of 7 Log CFU g^{-1} in TAMB is a limit of microbial quality (Senter et al., 2000). Using this parameter can be concluded that UV-C radiation had no effect on reducing the microbial load to indicate of a good quality because our results showed that all groups had values greater than 7 Log CFU g^{-1} after the 6^{th} day of storage. Similar results can be observed in *Enterobacteriacea* count, with the exception of medium and high UV-C intensities witch reach values above of 7 Log CFU g^{-1} after the 7^{th} day of storage time.

Our results are similar to that reported by others researches in different meats. Yndestad et al. (1972) evaluated the effect of 10 000 μW/cm² at 12.8 sec on the surface of the broiler carcass and observed that although was an immediate effect on the surface contamination. However, concluded that the UV-radiation does not have any prolonging effect on shelf life. On the other hand, Huang and Toledo (1982) showed a initial reduction of microbial count by 2-3 log on surface mackerel irradiated with 300 μW/cm² at 16.6 mim and 120-180 mW/cm² at 40 sec and consequently a prolonging the storage life.

The effect of UV-C radiation over bacterial count in the time can be varied by several factors. Deficiencies in the intensity-time relation can explain an initially retarded in the ability of microorganisms to grow and multiply but after some days of storage at 4±2°C these microorganisms could be recover their growth and multiplication abilities. Guerrero-Beltr·n and Barbosa-C·novas (2004) explained that the effect of UV radiation on microorganisms may vary from species to species and, in the same species, may depend on the strain, growth media, composition of the food, density of microorganisms and other characteristics. Another hypothesis is that the damage occurring at the DNA level could be repaired by protein factors (DNA repair genes), this phenomenon called photoreactivation by which inactivated microorganisms recover activity through the repair the dimer species (splitting of thymine and other pyridines) formed after the radiation process. (Nebot Sanz et al., 2007). It is important to remark the limited action of UV irradiation because only eliminated bacteria on the surface and when the storage time progress, the internal bacteria, which were not reached by the radiation, could be increase rapidly. Korhonen et al. (1981) evaluated the effect of brief exposure of high intensity UV light on microbial survival in beef and observed that irregular surface to provide a safe niche for bacteria to live by physically blocking the lethal effects of the UV light, could be affected the survival of bacteria on beef muscle at refrigerated

temperatures after exposure to UV light at varying times (90-120 sec) and intensities (400-1200 μW/cm²).

TBARS, pH and color

All groups followed a similar trend; it increased from 0.14-016 to 0.38-0.40 mg MDA/kg as a storage time progressed. Although a slight difference between the groups was appreciated, the statistics confirmed that the TBARS values of all groups did not differ significantly ($p>0.05$) at storage times up to 9 days (Table 2)

Our result for TBARS were similar that others researches that used UV-C radiation in chicken meats. Wallner-Pendleton et al. (1994) showed that after 10 days of storage at 7 °C, TBARS values of thigh chicken meat were 1.3 mg/kg compared with 1.7 mg/kg for controls. In the same way, Chun et al. (2010) observed that TBARS value of the chicken breasts slowly increased during storage, regardless of UV-C dose. The TBARS values of the irradiated chicken breasts after 6 d of storage were 0.97, 0.91, 1.05, 0.94, and 1.03 mg MDA/kg at 0, 0.5, 1, 3, and 5 kJ/m2 irradiation. Some authors related similar values of TBARS in storage time without any treatment. Lázaro et al. (2012) observed that different chicken meats gradually increasing during 18 days in refrigerated storage and did not exceeded 0.5 mg MDA/kg. Alasnier et al. (2000) determined low initial levels of lipid oxidation 0.03 $mg.kg^{-1}$followed by a linear increment until 0.30 $mg.kg^{-1}$ at day 14 in chicken breast meat. Castellini et al. (2006) evaluated the oxidative stability in organic chickens and determinate that the lowest values in TBARS during the storage time were due the higher antioxidant capacity, resulting from the higher antioxidant intake (α-tocopherol, carotenoids, etc.) but in our experiment do not evaluated the diet composition.

On the other hand, the pH values had a slightly variations through the storage time. The control and low intensity groups reduced the values from 6.5 and 6.4 to 6.3 and 6.1 respectively. In contrast, medium and high intensity groups had initial value of 6.3, followed irregular variations over the time but at the end of experiment values were similar to observed at the beginning (Table 2). Chun et al. (2010) showed that regardless of irradiation initial pH values were between 6.24-6.45, increased during storage. They observed a slight difference with no irradiated chicken meat after 6^{th} day, indicating that increasing irradiation dose leads to a smaller change in pH. The pH values can vary according several factors like a sex, age, rearing system, welfare, etc. Our results were slightly high during all storage time, even for

control group. Lázaro et al. (2012) observed initial values between 5.57 and 6 that increased in the storage time.

The lightness (L* values) decreased in all groups until the 5 and 6th day of storage, after that a slightly increasing was observed. The redness (a* values) were no significant affected by the UV-treated, all groups showed initial values between 17 and 19 which decreased to 15 after 3th day remaining constant until the end. The yellowness (b* values) showed some interesting changes. Control and low intensity groups presented the same trend during the experiment with initial values of 16 which decreased until 10 at the end of experiment. On the other hand, medium and high intensity groups maintained the yellow color almost constant during the storage (Table 3).

Wallner-Pendleton et al. (1994) observed a slight effect only on b color values in chicken meat samples increasing from 3.50 to 4.68 during 10 storage days. Lyon et al. (2007) observed a small effect on a* values 7 d after UV exposure. However, this change would probably not be large enough to be noticed visually Chun et al. (2010) determinate that only a* values showed a significant difference in all treatments after 6th day of storage. On the other hand, Haughton et al. (2011) demonstrated negligible changes in the color in chicken meat treated with UV-C light. They found that dose up to 0.192 J/cm² did not affect the Hunter L* or a* values of raw chicken, although changes in some of the b* values were significant. On the contrary, while examining the decontamination of unpackaged and vacuum-packaged boneless chicken breast with pulsed ultraviolet light Keklik et al. (2010) observed that color parameters did not change significantly after treatments.

Biogenic amines

Results of biogenic amines can be observed in Table 4. Initial concentrations of tyramine were in the range from 0.42 to 1.07 mg kg^{-1} for all groups, these values gradually increased until the end of experiment. However, the groups irradiated with medium and high intensities showed a significant increment after of 5th day, reaching final values of 10 and 9 mg kg^{-1} respectively, while the control and low intensity groups had final values of 6 mg kg^{-1}. All groups showed similar values of putrescina (0.17-0.86 mg kg^{-1}) and cadaverine (0.01-0.78 mg kg^{-1}) in the first 3 and 5 days of storage, respectively. After that a quickly increased was observed, reached values between 300-650 and 767-1029 mg kg^{-1} respectively at the end of experiment. The groups treated with UV light showed a significant increment of putrescina and cadaverine compared with the control group.

In all groups the concentration of spermine were constant or showed only a moderately increasing tendency, nevertheless a clearly difference could be observed between the control group with values of 2 mg kg^{-1} during the storage time and UV-C groups with values of 0.07-0.92 mg kg^{-1}. Spermidine values gradually increasing in control, low and medium intensity groups, whereas the maximum intensity group showed a slightly decreased of 1.94 to 1.23 mg kg^{-1}. Histamine values showed a gradually increase during the storage in all groups with a clear effect. Mediun and high intensity groups followed a similar trend with initial values of 0.46 and 1.01 mg kg^{-1} increased until 18 mg kg^{-1} in both groups to the end of experiment. On the other hand, control and low intensity groups showed initial values of 3.35 and 2.48 mg kg^{-1} increasing to 30 and 25 mg kg^{-1} respectively to the end.

No reference about the behavior of the amines in meat subjected to UV-C was found. However, the results found in our work can be compared with other authors who research the changes of biogenic amines as a quality indicator of ageing in meats. Our results were partially similar to reported by (Balamatsia et al., 2006) who observed a constant increasing in the chicken meat values of tyramine (from 0.02 to 4 mg kg^{-1}), putrescine (from 58 to 409 mg kg^{-1}), cadaverine (from 19 to 252 mg kg^{-1}) and histamine (from not determinate to 19 mg kg^{-1}) throughout the entire storage period (17 days) at 4°C. On the other hand, values of spermine and spermidine has a tendency of remain unaltered or gradually decreased, this fact is because these polyamines are naturally occurring amines in fresh meat and always are found at relatively constant level (Hernández-Jover et al., 1997, Ruiz-Capillas and Jiménez-Colmenero, 2004) or can be used as a nitrogen source by some microorganisms (Bardócz, 1995). Contradictory, this behavior was not observed in our experiment where both amines showed a slightly increased. In general, although specific biogenic amines are correlated to ageing and decomposition of proteinaceous foods, we results showed that they are not very sensitive as indicators of early ageing conditions of UV-C irradiated chicken meat.

The increment of biogenic amines after the UV exposition can be explained like a consequence of some events of cell stress in the muscle. Løvaas (1991) demonstrated that polyamines inhibit the oxidation of polyunsaturated fatty acids, and its antioxidative effect is correlated with the number of amine groups in the polyamine. The participation in cell membrane stabilization and cell proliferation and differentiation, since they are involved in DNA, RNA and protein synthesis, has been of extraordinary interest as polyamines (Stadnik and Dolatowski, 2010). Although the effect of UV-C irradiation on biogenic amines has not been studied, its effect has been reported in plants. Kondo et al. (2011) observed that level of

putrescina and spermine increased in apples after the UV-C irradiation, these authors suggested that polyamines may play a role in scavenging reactive oxygen and it are involved in protection against stress

Some authors try to explain the relationship of biogenic amines and the effect of other stressors in meat. Min et al. (2007) observed an increment in histamine, spermine and spermidine after gamma irradiation (2kGy) in chicken breast. They suggested that this increment is attributed at changes in tertiary structure of proteins due to the poultry meat contain substantial amounts of water, the radicals produced from water molecules activated by irradiation that can change the structure of proteins and change their physicochemical properties through fragmentation, cross-linking, coagulation, or oxidation. Other stress situation that can produce an increment of biogenic amines concentration is the freezing. Kozová et al. (2009) observed an increase of polyamines content after 6 months of frozen storage of chicken meat and hypothesized that a disruption of cells by freezing caused a release of polyamines bound to proteins or some other cell components.

The relation between bacterial count and biogenic amine production in poultry meat was observed by Rokka et al. (2004) observed a positive correlation between biogenic amines (putrecine, cadaverine and tyramine) and bacterial (Enterobacteriaceae and total aerobics mesophilic). The positive correlation between putrescine and E*nterobacteriaceae* was also observed in chicken meat products by Nassar and Emam (2002)). Values of putrescine and tyramine were proposed as the limit for spoilage initiation in precooked chicken meat stored aerobically after 8 days by Patsias et al. (2006), in this time total aerobics mesophilic count was 6.5 log CFU g^{-1}. Valero et al. (2005) determinate that levels cadaverine, putrescina and tyramine increased when the bacterial aerobic count was >6 Log CFU g^{-1} in pork meat

The UV-C technology is a non-thermal process which effect varies depending on the type of food. Due to the wide variety of organisms, including strains, the level of UV-C required for improve the bacteriological quality can vary according to initial bacterial load (Guerrero-Beltr·n and Barbosa-C·novas, 2004). According to Bintsis et al. (2000) it is evident that the food industry is faced with two conflicting pressures. On the one hand, there is the need to produce microbiologically safe food, while on the other; consumers are seeking foods with more natural flavours and textures. Consequently, a resurgence of interest in UVC could well be appropriate, for it does seem that UV radiation is one of the least exploited antimicrobial treatments for surfaces and, perhaps, foods themselves.

Conclusion

The high intensity UV-C light show interesting results for reduction of pathogenic bacteria and can be used as a non thermal procedure to ensure the superficial quality of packing poultry breast meat without important changes in biochemical parameters, nevertheless, the use of biogenic amines like a ageing indicator can be suspicious and expressing an inferior product quality, suggesting that this parameter is not suitable to evaluate chicken meat irradiated with UV-C. It is recommended more researches in order to determinate the effect of increasing the intensities and reduce the time of exposition to be possible the use of this technology in the production line

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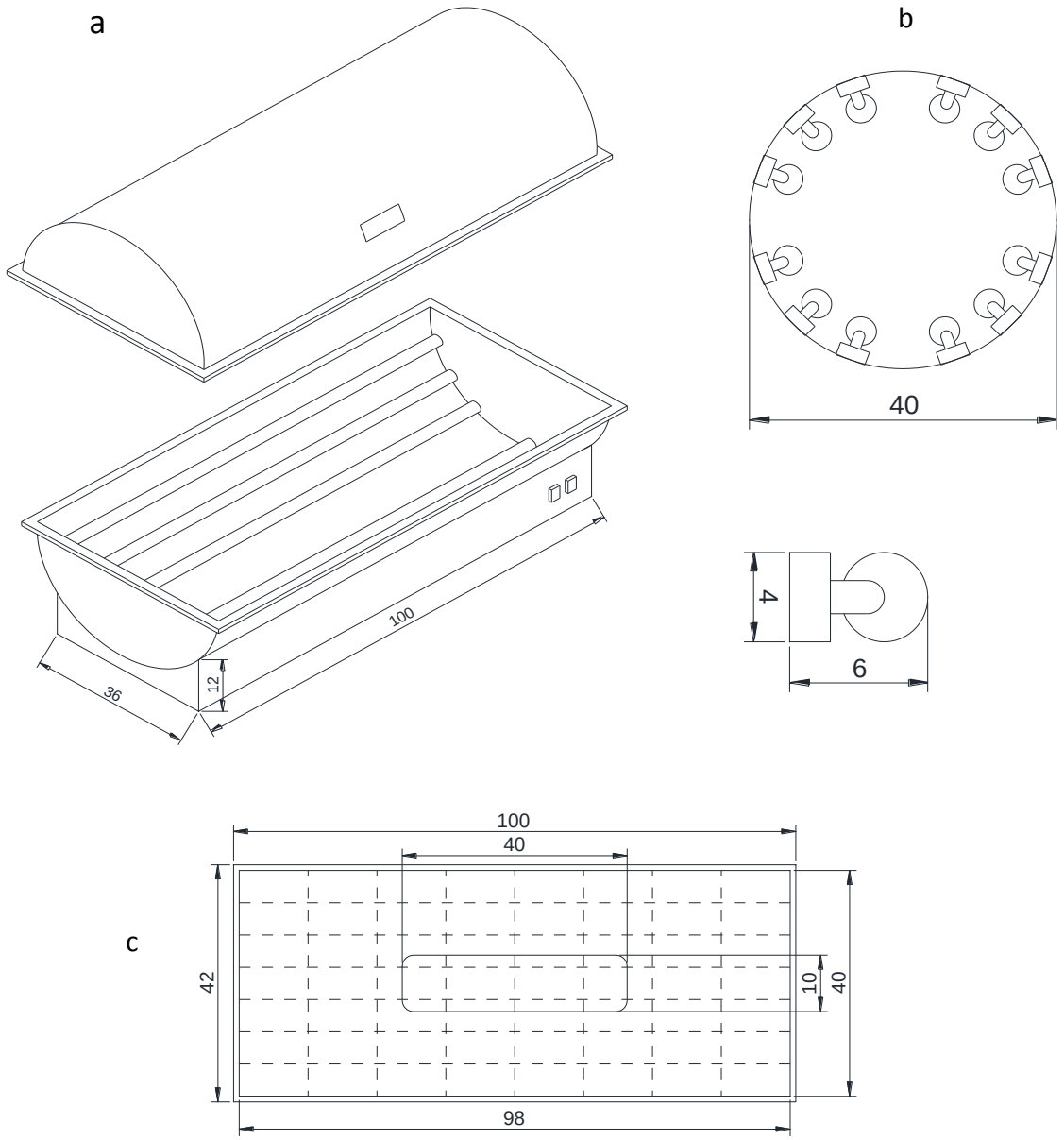
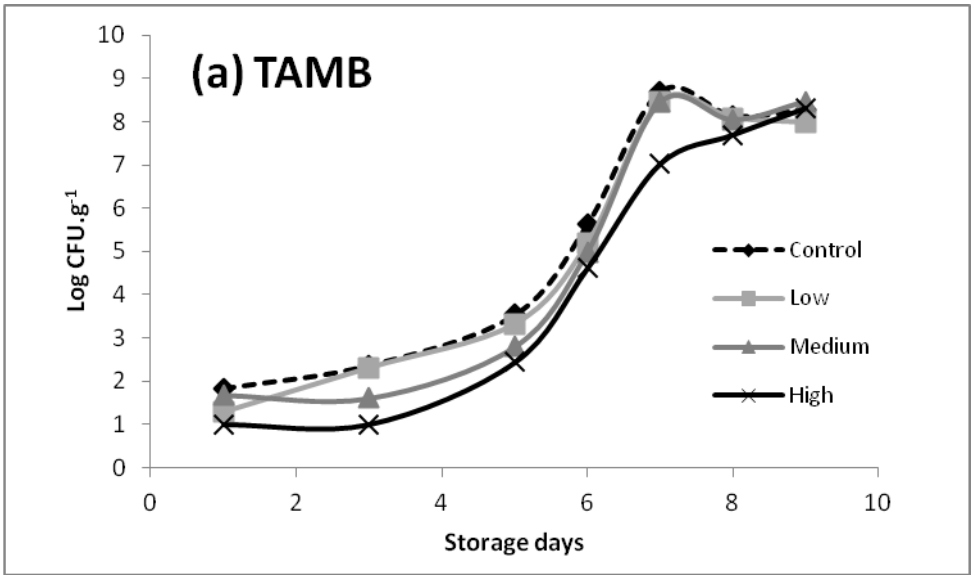


Figure 1. Layout of UV-C instrument. (a) Stainless-steel irradiation chamber. (b) Internal dispositions of UV-C lamps. (c) Nylon net used to put the samples. All dimension values in centimeters (cm)



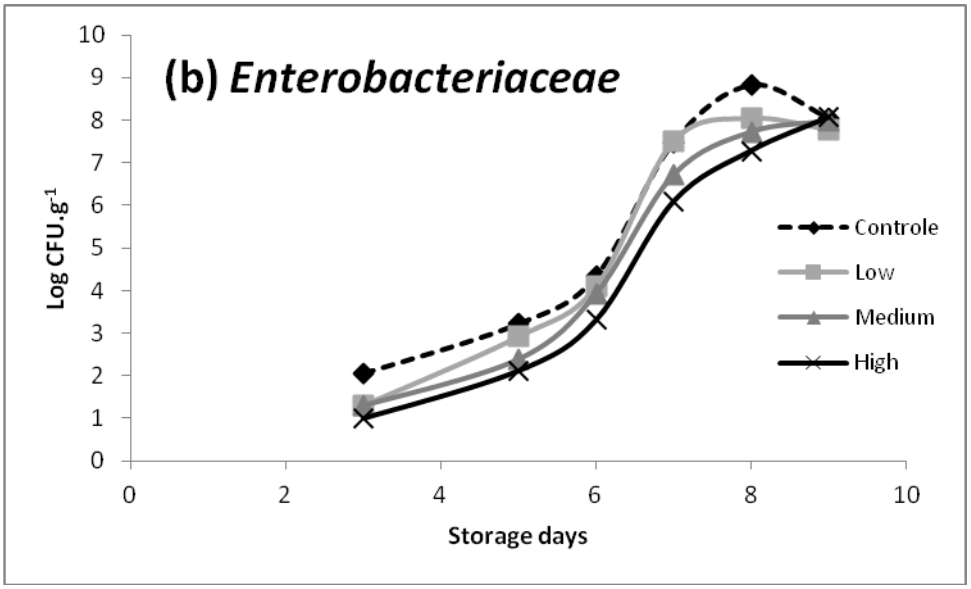


Figure 2: Behavior of TAMB (a) and Enterobacteriaceae (b) *a*fter the exposition of low, medium and high UV-C intensities and control groups during the storage time.

Table 1. Log reductions (CUF/g) of *Salmonella* serogrups inoculed in breast chicken meats*

Intensity	Treatment time (s)			
	30	60	90	120
L	0.14 ± 0.01 C	0.34 ± 0.03 B	0.38 ± 0.03 AB	0.48 ± 0.04 A
M	0.18 ± 0.04 B	0.28 ± 0.01 AB	0.33 ± 0.07 AB	0.36 ± 0.02 A
H	0.34 ± 0.03 B	0.42 ± 0.02 B	0.60 ± 0.03 A	0.57 ± 0.01 A

* At the same intensity, values not followed by the same letter are significantly different ($P < 0.05$).

Abbreviations: C = control group, L = Low UV-C intensity (0.62 mW/cm²), M = Medium UV-C intensity (1.13 mW/cm²), High UV-C intensity (1.95 mW/cm²).

Table 2. pH and TBARS values of chicken meat submitted to UV-C irradiation during 9 days of storage time.

Group	Storage days						
	0	3	5	6	7	8	9
pH							
C	6.5 ± 0.0Aa	6.0 ± 0.0Bd	6.1 ± 0.0Bc	6.2 ± 0.0Bc	6.2 ± 0.0Bc	6.4 ± 0.0Aab	6.3 ± 0.1Ab
L	6.4 ± 0.0Aa	6.3 ± 0.0Abc	6.1 ± 0.0Bd	6.2 ± 0.0Bc	6.3 ± 0.0Aab	6.4 ± 0.0Aa	6.1 ± 0.0Bc
M	6.3 ± 0.0Bb	5.9 ± 0.0Bd	6.2 ± 0.0Ac	6.3 ± 0.0Ab	6.3 ± 0.0Ab	6.3 ± 0.0Bb	6.4 ± 0.0Aa
H	6.3 ± 0.0Ba	6.3 ± 0.0Aa	6.2 ± 0.0Ab	6.2 ± 0.0Bb	6.1 ± 0.0Cc	6.0 ± 0.0Cc	6.3 ± 0.0Aa
TBARS (mg MDA/kg)							
C	0.14 ± 0.01Acd	0.11 ± 0.01Bd	0.13 ± 0.01Ad	0.19 ± 0.01Bc	0.27 ± 0.01Ab	0.30 ± 0.01Ab	0.36 ± 0.03Aa
L	0.14 ± 0.01Ad	0.17 ± 0.02Adc	0.14 ± 0.01Ad	0.21 ± 0.01Bc	0.28 ± 0.00Ab	0.31 ± 0.01Ab	0.38 ± 0.02Aa
M	0.16 ± 0.02Ad	0.18 ± 0.01Ad	0.16 ± 0.02Ad	0.26 ± 0.01Ac	0.30 ± 0.01Ab	0.32 ± 0.00Ab	0.39 ± 0.01Aa
H	0.16 ± 0.01Ac	0.19 ± 0.02Ac	0.16 ± 0.01Ac	0.27 ± 0.01Ab	0.31 ± 0.04Ab	0.34 ± 0.01Aab	0.40 ± 0.02Aa

Different letters in the same row and different numbers in the same column represent significant differences ($p < 0.05$).

Abbreviations: TBARS = Thiobarbituric Acid Reactive Substances, C = control group, L = Low UV-C intensity (0.62 mW/cm²), M = Medium UV-C intensity (1.13 mW/cm²), High UV-C intensity (1.95 mW/cm²).

Table 3. Cie Lab color values of chicken meat submitted to UV-C irradiation during 9 days of storage time.

Group	Storage days						
	0	3	5	6	7	8	9
L*							
C	55.66 ± 0.19Ba	53.31 ± 1.74Aab	53.43 ± 0.92Aab	50.93 ± 2.67Ab	52.53 ± 1.46Bab	55.20 ± 0.62Aa	54.12 ± 1.78ABab
L	56.51 ± 1.04ABa	53.12 ± 0.62Ab	51.21 ± 1.30Ab	51.16 ± 0.68Ab	56.91 ± 1.03Aa	54.37 ± 1.01Aa	55.47 ± 1.27Aa
M	57.20 ± 2.43ABa	55.67 ± 0.96Aa	53.98 ± 1.12Aab	52.33 ± 0.50Ab	51.27 ± 1.54Bb	51.34 ± 2.71Bb	52.68 ± 0.73Bab
H	58.30 ± 1.53Aa	53.96 ± 1.59Ab	53.83 ± 0.92Ab	53.35 ± 0.58Ab	51.91 ± 1.36Bb	53.88 ± 0.68ABb	53.36 ± 0.31Bb
a*							
C	18.35 ± 1.02Aa	15.59 ± 0.64Ab	15.76 ± 0.26Ab	16.02 ± 0.61Ab	15.74 ± 0.43Bb	14.66 ± 1.05Bb	15.43 ± 0.25Bb
L	17.68 ± 0.75Aa	15.74 ± 0.82Aab	15.10 ± 0.79Ab	15.28 ± 0.39Ab	15.26 ± 0.76Bb	14.20 ± 1.58Bb	15.28 ± 0.30Bb
M	19.01 ± 0.48Aa	15.70 ± 0.35Ab	16.52 ± 0.70Ab	15.09 ± 0.85Ab	16.94 ± 1.45ABb	16.63 ± 0.93Ab	16.27 ± 0.14Ab
H	18.20 ± 1.67Aa	15.94 ± 0.71Ab	16.42 ± 0.82Aab	15.40 ± 0.51Ab	16.98 ± 0.25Aab	16.36 ± 1.03Aab	15.57 ± 0.29Bb
b*							
C	16.78 ± 0.54Aa	15.27 ± 0.70Ab	14.00 ± 0.55Bb	12.58 ± 0.73Bb	13.60 ± 0.94Ab	9.68 ± 0.05Bc	10.22 ± 0.51Cc
L	16.08 ± 0.22ABa	16.78 ± 0.22Aa	16.29 ± 0.15Aa	15.76 ± 0.97Aa	14.45 ± 0.35Ab	10.80 ± 0.98Bc	10.00 ± 0.24Cc
M	14.72 ± 0.48Ca	14.19 ± 0.91Aab	13.69 ± 0.56Ba	13.90 ± 0.98ABa	12.20 ± 0.49Bb	12.75 ± 0.81Aab	12.55 ± 0.15Bb
H	15.40 ± 0.51BCa	15.25 ± 0.73Aa	14.37 ± 0.12Aa	15.60 ± 0.68Ab	13.62 ± 0.79Ab	13.79 ± 0.55Ab	13.37 ± 0.64Ab

Different letters in the same row and different numbers in the same column represent significant differences ($p < 0.05$).

Abbreviations: C = control group, L = Low UV-C intensity (0.62 mW/cm²), M = Medium UV-C intensity (1.13 mW/cm²), High UV-C intensity (1.95 mW/cm²).

Table 4. Biogenic amines values of chicken meat submitted to UV-C irradiation during 9 days of storage time.

Group	Storage days						
	0	3	5	6	7	8	9
Tyramine							
C	0.42 ± 0.54Bd	0.23 ± 0.06Cd	3.17 ± 0.91Ac	2.74 ± 0.04Bc	3.16 ± 0.16Cbc	3.74 ± 0.62Bbc	6.94 ± 0.78Ba
L	0.49 ± 0.12Bd	0.67 ± 0.03Bd	2.30 ± 0.18Ac	2.67 ± 0.62Bc	3.75 ± 0.03Cb	3.50 ± 0.17Bb	6.70 ± 0.43Ba
M	0.51 ± 0.02Bd	0.53 ± 0.00Bd	2.68 ± 0.41Ac	5.91 ± 0.13Ab	7.42 ± 1.25Ab	10.20 ± 0.15Aa	10.76 ± 1.33Aa
H	1.07 ± 0.05Ac	1.08 ± 0.28Ac	4.23 ± 0.93Ab	5.80 ± 0.04Ab	5.58 ± 0.40Bb	9.39 ± 0.45Aa	8.99 ± 1.39Aa
Putrescine							
C	0.32 ± 0.01Af	0.46 ± 0.18Af	5.18 ± 0.30Ae	15.68 ± 0.42Cd	275.50 ± 3.46Ab	255.41 ± 7.81Bc	304.49 ± 2.81Da
L	0.69 ± 0.06Af	0.37 ± 0.04Af	4.10 ± 0.15Ae	123.37 ± 0.52Bd	139.52 ± 0.32Cc	144.47 ± 0.35Db	537.10 ± 1.27Ca
M	0.65 ± 0.15Ae	0.17 ± 0.00Af	6.64 ± 0.25Ad	141.45 ± 7.16Ac	196.15 ± 0.23Bb	196.15 ± 0.23Cb	646.19 ± 2.35Aa
H	0.77 ± 0.09Af	0.86 ± 0.25Af	6.38 ± 0.20Ae	17.03 ± 0.15Cd	66.51 ± 12.98Dc	320.93 ± 2.18Ab	577.78 ± 2.85Ba
Cadaverine							
C	0.01 ± 0.01Af	0.36 ± 0.29Ae	0.78 ± 0.17Ae	67.10 ± 1.36Dd	643.73 ± 5.62Bc	723.29 ± 15.26Bb	767.13 ± 4.18Da
L	0.11 ± 0.13Ad	0.01 ± 0.00Ae	0.29 ± 0.06Ad	355.43 ± 1.27Bc	515.27 ± 17.32Cb	549.93 ± 12.20Cb	894.44 ± 0.68Ca
M	0.05 ± 0.07Ad	0.01 ± 0.00Ad	0.02 ± 0.01Ad	457.70 ± 1.87Ac	693.03 ± 2.74Ab	701.58 ± 6.15Bb	1084.11 ± 9.77Aa
H	0.01 ± 0.01Af	0.10 ± 0.11Af	0.36 ± 0.03Ae	217.19 ± 15.59Cd	503.20 ± 3.81Cc	892.98 ± 19.12Ab	1029.60 ± 24.86Ba
Spermidine							
C	0.47 ± 0.02Bc	0.66 ± 0.26Cc	2.04 ± 0.08Ab	2.91 ± 0.03Aa	2.05 ± 0.65Cb	1.97 ± 0.28Cb	2.12 ± 0.18Cb
L	2.08 ± 0.37Ae	2.70 ± 0.01Ac	2.11 ± 0.10Ae	2.55 ± 0.01Ad	3.91 ± 0.18Ab	4.34 ± 0.26Aab	4.71 ± 0.04Aa
M	2.03 ± 0.12Ac	2.22 ± 0.01Ab	2.11 ± 0.01Ac	2.35 ± 0.06Aab	2.94 ± 0.21Ba	2.96 ± 0.11Ba	2.83 ± 0.49Ba
H	1.94 ± 0.01Aa	1.46 ± 0.08Bc	1.64 ± 0.04Bb	1.64 ± 0.16Bbc	1.73 ± 0.25Cbc	1.11 ± 0.01Dd	1.23 ± 0.14Dd
Histamine							
C	3.35 ± 0.06Ad	5.61 ± 0.57Ac	6.06 ± 0.04Ac	6.04 ± 0.03Bc	17.17 ± 1.56Ab	27.67 ± 0.91Aa	30.07 ± 1.22Aa
L	2.48 ± 0.28Ae	5.68 ± 0.02Ad	6.14 ± 0.13Ac	6.48 ± 0.10Ac	15.26 ± 0.75Ab	17.89 ± 0.29Bb	25.65 ± 2.40Ba
M	0.46 ± 0.16Cd	1.82 ± 0.08Bc	2.04 ± 0.04Bc	5.89 ± 0.10Cb	5.52 ± 0.16Bb	17.55 ± 1.72BCa	18.84 ± 0.22Ca
H	1.01 ± 0.04Bf	1.66 ± 0.41Be	2.33 ± 0.21Be	3.06 ± 0.00Dd	5.41 ± 0.00Bc	15.59 ± 0.42Cb	18.77 ± 0.95Ca

Spermine							
C	2.02 ± 0.01A	2.02 ± 0.01Ac	2.14 ± 0.03Ab	2.13 ± 0.05Ab	2.17 ± 0.06Ab	1.98 ± 0.22Ac	2.92 ± 0.18Aa
L	0.37 ± 0.15Bbc	0.02 ± 0.00Bd	0.36 ± 0.06Bc	0.37 ± 0.23Cbc	0.25 ± 0.14Cc	0.45 ± 0.07BCb	0.74 ± 0.08Ba
M	0.10 ± 0.00Cd	0.01 ± 0.00Be	0.26 ± 0.01Cc	0.82 ± 0.11Ba	0.92 ± 0.03Ba	0.75 ± 0.28Bab	0.66 ± 0.08Bb
H	0.07 ± 0.00Cc	0.01 ± 0.01Bc	0.15 ± 0.04Db	0.11 ± 0.00Cb	0.35 ± 0.05Ca	0.34 ± 0.04Ca	0.41 ± 0.01Ca

Different letters in the same row and different numbers in the same column represent significant differences ($p < 0.05$).

Abbreviations: C = control group, L = Low UV-C intensity (0.62 mW/cm²), M = Medium UV-C intensity (1.13 mW/cm²), High UV-C intensity (1.95 mW/cm²).

4 CONSIDERAÇÕES FINAIS

O método proposto permitiu a identificação simultânea das aminas biogênicas por CLAE em carne de frango com as vantagens de uma fácil extração, efetiva derivatizacão e alta resolução em um tempo curto, utilizando um sistema isocrático de acetonitrilo e água, alem disso foi obtida boa seletividade, linearidade, precisão, recuperação, robustez e limites de detecção e de quantificação.

Nas cinco carnes de aves avaliadas foram observados diferentes comportamentos durante o período de estocagem, sendo que a tiramina, putrescina e cadaverina são as aminas que poderiam ser utilizadas como indicadores de deterioração. Isto foi confirmado pelas técnicas quimiométricas o que enfatiza a necessidade de utilizar modelos quimiométricos e considerar todas as possíveis relações com as variáveis em estudo.

Valores de pH e putrescina tiveram um incremento progressivo nos cinco tipos de carne de aves, alem disso a cadaverina também foi incrementada em carne de codorna e frangos orgânico e caipira pelo que poderiam servir como indicadores de deterioração após os 12 dias de estocagem. Valores de TBARS parecem não ter relação com a deterioração devido a que se incrementou só no primeiro dia de estocagem.

Na utilização da luz UV-C foram evidenciados resultados positivos na redução de bactérias patógenas, podendo ser utilizada como um procedimento não térmico para garantir a qualidade superficial em peitos de frango embalados sem alterar parâmetros bioquímicos. Contudo, a utilidade das aminas biogênicas como indicadores de deterioração é limitada e pode expressar uma qualidade inferior do produto pelo que se sugere que este parâmetro não e o mais adequado para avaliar este tipo de carnes.

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6 APÊNDICES

6.1 PAPER I

Validation of an HPLC Methodology for the Identification and Quantification of Biogenic Amines in Chicken Meat

César A. Lázaro, Carlos A. Conte-Júnior, Fernanda L. Cunha, Eliane T. Márisco, Sérgio B. Mano & Robson M. Franco

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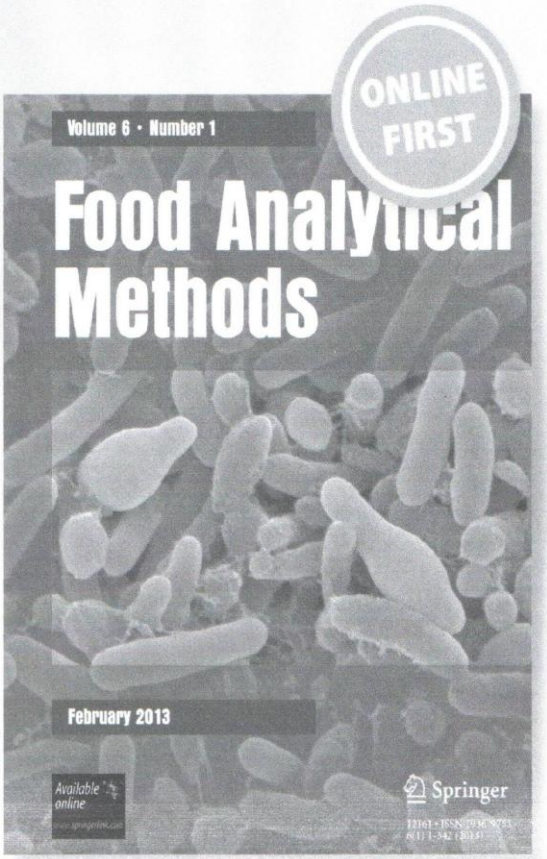

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Validation of an HPLC Methodology for the Identification and Quantification of Biogenic Amines in Chicken Meat

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Abstract This study validated a high performance liquid chromatography (HPLC) method to determine biogenic amines in chicken meat. For the identification of biogenic amines, an isocratic elution system coupled with a UV detector (254 nm) was used after a perchloric acid (5 %) extraction and benzoyl chloride derivatization of the samples. The standards of tyramine, putrescine, cadaverine, spermidine, and spermine were used for the following validation parameters: selectivity, linearity, accuracy, recovery, limit of detection and quantification, and robustness. Finally, chicken meat commercialized in two types of packaging was evaluated. The results showed an excellent selectivity and separation of all amines, $r^2>0.99$, relative standard deviation <5 %, recovery between 64 and 112 %, limits of detection and quantification between 0.03–1.25 and 0.15–5.00 $\mu g L^{-1}$, respectively, and appropriate robustness for the proposed methodology. Moreover, both chicken meat commercial packages had similar values for all amines; only tyramine was significantly different ($P \leq 0.05$). The proposed method was suitable to detection and quantification of simultaneous five biogenic amines in chicken meat.

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Introduction

The Brazilian production of chicken meat in 2010 reached 12,230 million tons, a growth of 11.38 % in comparison with 2009; this increase was mainly due to internal consumption and exportation. With this performance, Brazil approaches China, currently the second largest chicken producer in the world at 12,550 million tons, only below the USA at 16,648 million tons, according to projections of the Department of Agriculture of the United States (ABEF 2011).

Due to increases in the global demand for chicken meat, poultry suppliers are obliged to implement specific controls to guarantee food safety and a high-quality product. The chicken meat is especially susceptible to protein degradation, and the determination of substances that originate by this process can be used as quality indicators in meat (Dadáková et al. 2009). Biogenic amines are low-molecular-weight substances, primarily produced by amino acid decarboxylase enzymes produced by some microorganisms. The presence of these molecules in foods is directly related to amino acid composition, microflora, storage temperature, maturation time, packing, and other factors (Halász et al. 1994).

Reliable methods for evaluating biogenic amine production are important for preventing food-borne intoxication, maintaining good control of the production chain,

and checking the safety quality. High performance liquid chromatography methods are reliable and highly sensitive techniques for the simultaneous detection and quantification of different biogenic amines (EFSA 2011). Some authors explored the advantage of this method in poultry meats (Buňková et al. 2010; Balamatsia et al. 2006; Baston et al. 2008; Gallas et al. 2010; Silva & Gloria 2002); nevertheless, the complete explanation about the technique and validation procedures is not fully detailed.

Validation is a process that is used to authenticate that the analytical procedure employed for a specific test is suitable for its intended use (Shah et al. 2000). In addition, it confirms good performance, reduces analytical errors, and improves the quality, reliability and reproducibility of the method under consideration (EURACHEM 1998). New analytical methods need to be validated in an objective way to demonstrate their application for predetermined usefulness (ISO 1999). Nonvalidated methods can generate unreliable results and are not officially recognized by national and international authorities (Baston et al. 2008).

Trustworthy results are a prerequisite for the interpretation and correct evaluation of scientific research and routine laboratory analyses. Unreliable results will over- or underestimate the possible effects of analyzed metabolites and lead to false interpretations and unjustified conclusions. For this reason, the aim of this study was to validate a methodology for biogenic amine determination in chicken meat using high performance liquid chromatography.

Material and Methods

Standards Preparation

Standards of tyramine ($C_8H_{11}NO$), putrescine ($C_4H_{12}N_2$), cadaverine ($C_5H_{14}N_2$), spermidine ($C_{14}H_{47}N_6O_{12}P_3$), and spermine ($C_{10}H_{26}N_4$) were purchased from Sigma-Aldrich® (St. Louis, MO, USA). Stock solutions for each amine (40 $\mu g L^{-1}$) were prepared in 0.1 N HCl and stored at 4±1 °C. For each validation phase, stock solutions were diluted with Milli-Q water (Simplicity UV, Millipore, Molsheim, France) and alkalinized with 2 N NaOH until pH>12 was reached. Samples were derivatized using benzoyl chloride (40 µL), homogenized (vortex, 15 s), and kept at room temperature for 20 min. The mixture was extracted two times with 1,000 µL of diethyl ether. The ether layer was aspirated and evaporated to dryness under a stream of nitrogen (Sample Concentrator Techne®, Cambridge, UK). Finally, the residue was dissolved in 1,000 µL of the mobile phase and stored at 4±1 °C.

Sample Preparation

Two commercial packages (an expanded polystyrene tray and a low-density polyethylene bag) of frozen chicken breasts (*Gallus gallus domesticus*) were purchased (n=10 for each package) at markets in Rio de Janeiro, Brazil, and transported to the laboratory in insulated polystyrene boxes on ice. For biogenic amine extraction, 5 g of minced chicken meat was homogenized with 5 mL of 5 % perchloric acid. The homogenates were kept under refrigeration (4±2 °C) for 1 h and shaken continuously (Certomat® MV, B. Braun Biotech International, Melsungen, Germany); then, the mixture was centrifuged at 503×g for 10 min at 4±1 °C (Hermle Z 360 K) and filtered through Whatman no. 1 filter paper. The filtrates were neutralized (pH>6) with 2 N NaOH and kept in an ice bath (0±2 °C) for approximately 20 min, followed by a second filtration, and addition of NaOH (pH>12) under the same conditions. The derivatization procedure was carried out in the same way as for the standards.

Chromatographic Conditions

The chromatographic system consisted of a LC/10AS pump coupled to a SPD/10AV UV–Vis detector and a C-R6A chromatopack integrator (Shimadzu, Kyoto, Japan). Biogenic amine separations were performed on a Teknokroma Tracer Extrasil ODS2 (15×0.46 cm id., 5 µm) column equipped with a Supelco Ascentis C18 (2×0.40 cm id., 5 µm) guard column, under isocratic conditions. The mobile phase was prepared by mixing acetonitrile (Tedia®) and Milli-Q water, 42:58 (v/v); the mixture was degassed in an ultrasonic bath (Cleaner USC 2800 A). The flow rate was 1 mL min^{-1}, the injected volume was 20 µL, the column temperature was 20 °C, and the detector wavelength was set at 198 nm. Injections were performed using a 50-µL syringe (Hamilton Microliter TM 705), and the total run time was 15 min. An injection of pure acetonitrile for 10 min was used between each sample to flush the HPLC system. The biogenic amines were identified by retention time and were quantified by peak area.

Validation Parameters

The method for the identification of biogenic amines in chicken meat was validated in terms of the analytical parameters of selectivity, linearity, precision, recovery, limit of quantification, limit of detection, and robustness following conventional protocols from international guidelines (AOAC 2002; ICH 1995).

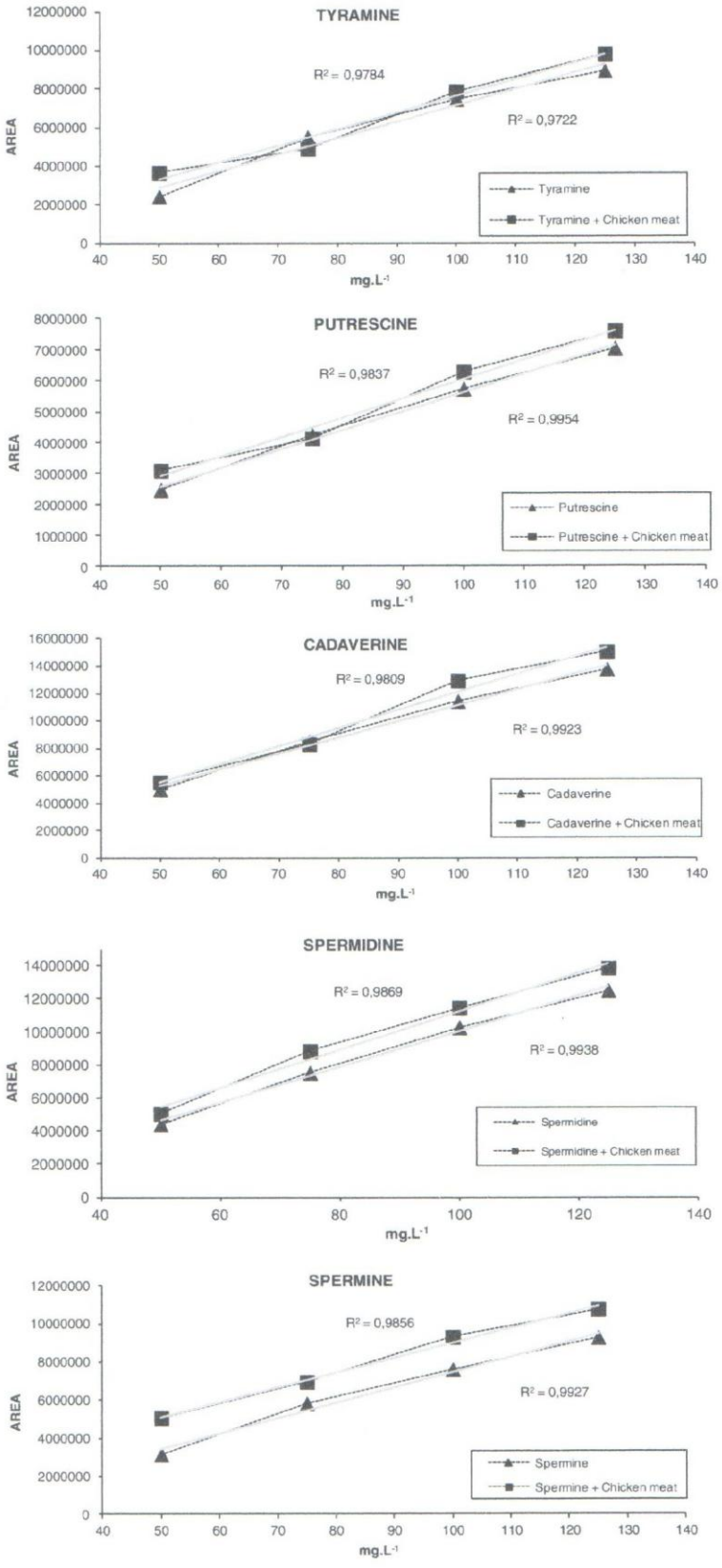

Fig. 1 Calibration curves for chicken meat samples spiked with amine standard and amine standard solution

Selectivity was performed injected different concentrations (50, 75, 100, and 125 mgL^{-1}) of each biogenic amine standard and compared with a solution of chicken meat spiked with the same biogenic amine standard concentration. Likewise, a mixed solution of five biogenic amines, a chicken meat sample, and a chicken meat sample spiked with the mix solution were injected and evaluated the retention times and the separation of each amine.

Linearity was determinate injected eight sample concentrations (1, 10, 12.5, 25, 50, 100, 125, and 250 mg L^{-1}) of each amine standard in three times onto the HPLC. Linear calibration curves were constructed, and the regression equation and the regression coefficient (r^2) were calculated for each biogenic amine.

Accuracy was considered at two levels: (1) repeatability: it was established with three different concentrations of amine mix standard: solution A (100 mgL^{-1} of putrescine, cadaverine, spermidine, spermine, and 300 mg L^{-1} of tyramine); solution B (200 mgL^{-1} of putrescine, cadaverine, spermidine, spermine, and 500 mg L^{-1} of tyramine); and solution C (250 mgL^{-1} of putrescine, cadaverine, spermidine, spermine, and 1,000 mgL^{-1} of tyramine), injected ten times and expressed as the mean, standard deviation (SD), and relative standard deviation (RSD); and (2) intermediate precision, performed with a mix solution (200 mgL^{-1} of putrescine, cadaverine, spermidine, spermine, and 400 mgL^{-1} of tyramine) injected five times. This procedure was repeated on three consecutive days and expressed in the same way for repeatability.

Recovery was tested by the standard addition procedure of injecting three amine concentrations (100, 150, and

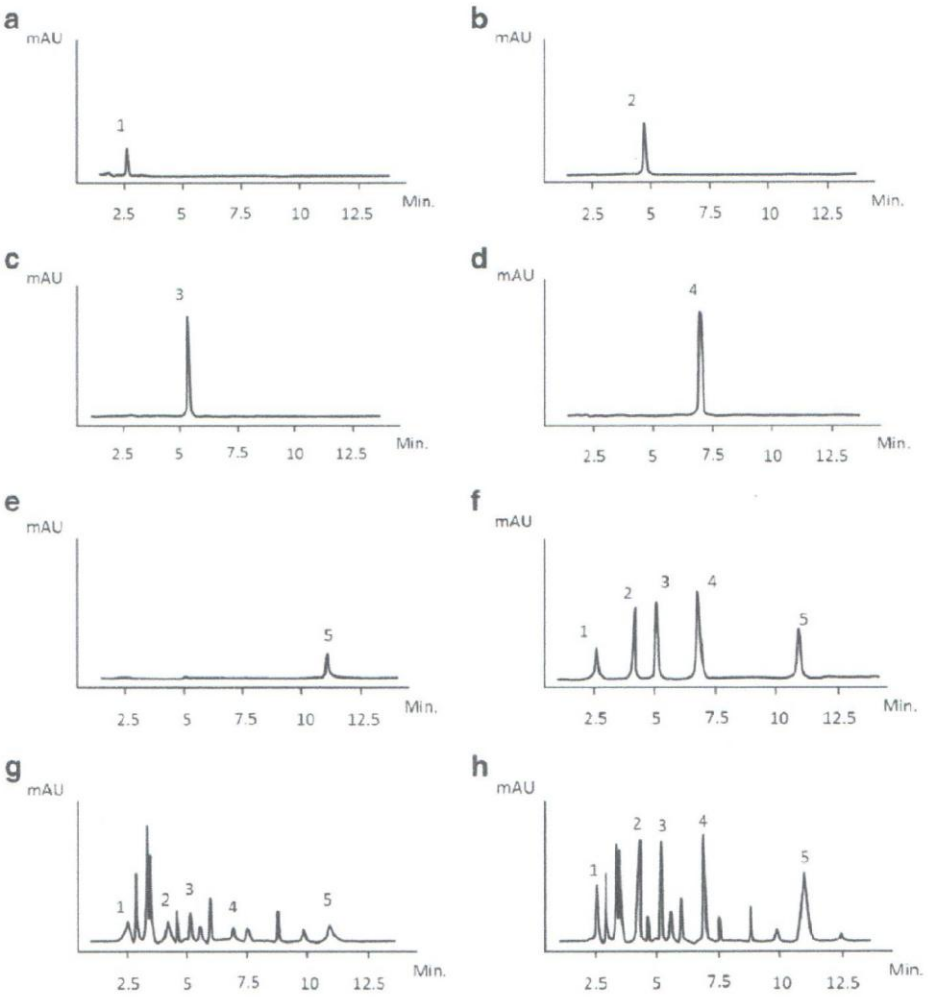

Fig. 2 Chromatograms of each amine standard (**a**, **b**, **c**, **d**, **e**), amine standard mixture (**f**), meat chicken sample (**g**), and meat chicken sample spiked with amine standard mixture (**h**). Peak identification: (*1*) tyramine, (*2*) putrescine, (*3*) cadaverine, (*4*) spermidine, and (*5*) spermine

Table 1 Linearity of proposed chromatographic method for biogenic amine determination, obtained with eight different concentrations between 1 and 250 mgL^{-1}

Biogenic amines	Regression equation	Regression coefficient (r^2)
Tyramine	Y=26297X−41220	0.9981
Putrescine	Y=42896X+16098	0.9977
Cadaverine	Y=48731X+43416	0.9997
Spermidine	Y=72733X−455722	0.9921
Spermine	Y=44107X−66297	0.9985

200 mgL^{-1}). The following equation, $R=[(C-A)/B]\times 100$, was used with chicken meat samples (A), standard amines (B), and meat chicken samples fortified with standard amines (C), prepared and injected in triplicate.

The determination of limit of detection (LOD) was based on visual evaluation. For this purpose, smaller concentrations of each amine calibration curve were injected in a decreasing sequence until the chromatographic signal reached an area that could be visually differentiated from the signal noise (baseline) at the lowest attenuation. When this area was identified, three injections of each solution were performed for confirmation. Limit of quantification (LOQ) was calculated in the same way as LOD.

The evaluation of robustness was planned with small variations in four parameters: mobile phase of acetonitrile/water (42:58, 43:57, and 41:59); wavelength (197, 198, and 199 nm); flow rate (0.95, 1.00, and 1.05 mL min^{-1}), and derivatization time (15, 20, and 25 min). For this purpose, a mix solution of putrescine, cadaverine, spermidine and spermine (200 mgL^{-1}), and tyramine (400 mgL^{-1}) was used.

Statistical Analysis

Data collected in this study were analyzed using GraphPad Prism® 5.00 package (GraphPad 2007) for windows by one-way ANOVA, and the means were compared with Tukey test ($P<0.05$).

Results and Discussion

Calibration curves of biogenic amine standards and chicken meat samples spiked with the amine standards were compared (Fig. 1). The curves were parallel, suggesting that there was no matrix interference. Food samples may contain components that interfere with performance measurement and may increase or decrease the signal detector (Shah et al. 2000). Additionally, the chromatograms for each amine standard, amine mix solution, chicken meat, and spiked chicken meat showed no interference at their respective retention times (tyramine, 2.32; putrescine, 4.35; cadaverine, 5.25; spermidine, 7.12; and spermine, 11.70). The separation of amines from other components in the matrix was effective (Fig. 2). This fact can be explained by the separation ability of the ODS2 column (15×0.46 cm id., 5 μm). The same stationary phase was used by Baston et al. (2008) who reported a good separation of amine standards in chicken meat samples. Errors of determination in samples can be considered a problem of analyte detection or low efficiency in the extraction. This result confirms the selectivity of the proposed method (EURACHEM 1998).

The regression equation and regression coefficient (r^2) can be observed in the Table 1. The r^2 values were

Table 2 Results of accuracy (repeatability) for the chromatographic method evaluated with three different concentrations of an amine standard mix solution

Biogenic amines	Mix A			Mix B			Mix C		
	Mean±SD	RSD	RT	Mean±SD	RSD	RT	Mean±SD	RSD	RT
Tyramine	315.81±3.51	1.11	2.47	470.32±5.78	1.23	2.50	1,038.51±13.32	1.28	2.52
Putrescine	74.55±1.07	1.43	4.36	167.60±2.55	1.52	4.32	229.40±0.59	0.26	4.38
Cadaverine	101.77±2.47	2.43	5.29	201.09±2.57	1.28	5.26	274.30±5.30	1.93	5.24
Spermidine	82.86±1.06	1.28	7.14	169.99±1.53	0.90	7.12	230.65±3.92	1.70	7.11
Spermine	92.67±0.08	0.09	11.65	185.84±2.70	1.45	11.68	227.80±2.47	1.08	11.62

Mix A: Putrescine, cadaverine, spermidine and spermine (100 mgL^{-1})+tyramine (300 mgL^{-1}); mix B: Putrescine, cadaverine, spermidine and spermine (200 mgL^{-1})+tyramine (500 mgL^{-1}); mix C: Putrescine, cadaverine, spermidine and spermine (250 mgL^{-1})+tyramine (1,000 mgL^{-1})

SD standard deviation, *RSD* relative standard deviation, *RT* retention time

Table 3 Results of accuracy (intermediate precision) for the chromatographic method evaluated with the same amine standard mix solution on three different days

Biogenic amines	Day 1			Day 2			Day 3		
	Mean±SD	RSD	RT	Mean±SD	RSD	RT	Mean±SD	RSD	RT
Tyramine[a]	412.02±6.76 a	1.64	2.52 a	415.52±11.61 a	2.79	2.46 a	416.89±30.18 a	7.24	2.48 a
Putrescine[b]	159.90±2.50 a	1.56	4.38 a	159.03±2.27 a	1.43	4.34 a	160.96±2.74 a	1.70	4.34 a
Cadaverine[b]	178.42±2.05 b	1.15	5.30 a	183.98±2.27 a	1.23	5.26 a	181.41±5.67 a	3.12	5.28 a
Spermidine[b]	179.47±9.44 a	5.26	7.12 a	180.98±5.32 a	2.94	7.10 a	182.13±6.45 a	3.54	7.08 a
Spermine[b]	172.70±6.00 a	3.47	11.68 a	168.04±5.24 a	3.12	11.60 a	173.18±5.17 a	2.99	11.64 a

Different letters in rows represent significantly different averages ($P \leq 0.05$)

SD standard deviation, *RSD* relative standard deviation, *RT* retention time

[a] Concentration: 400 mgL^{-1}

[b] Concentration: 200 mgL^{-1}

between 0.9997 and 0.9921 which are compatible with an optimal setting. These results were possible due to the use of eight different concentrations for the construction of the calibration curve, which showed a best-fit linear regression model. The European Community recommends at least five concentration levels for the construction of calibration curves (Paschoal et al. 2008). The results are consistent with Brazilian legislation and INMETRO, which consider 0.99 and 0.90 excellent r^2 values, respectively (INMETRO 2011; ANVISA 2003). Other researchers show an optimal data adjustment for the regression coefficient (r^2=0.99) for the same amines (tyramine, putrescine, cadaverine, spermine. and spermidine) (Baston et al. 2008; Tamim and Doerr 2003). The method developed was linear for the concentration range of 1–250 mgL^{-1}, with r^2 values above 0.99 for all of the amines studied.

The RSD values were less than 2.5 % in the three concentrations of standard mix solution used for repeatability (Table 2). In assessing the accuracy, previous literature had determined that an RSD equal to or less than 5 % was an acceptable value for bioanalytical methods (ANVISA 2003), and up to 20 % can be considered in methods for quantifying trace elements (Ribani et al. 2004). These results were similar to a previous research, with values for RSD less than 3.87 % for the identification of biogenic amines in chicken meat (Tamim and Doerr 2003).

For intermediate precision, the RSD results showed no significant difference ($P>0.05$) for tyramine, putrescine, spermidine, and spermine on three different days (Table 3). However, cadaverine values were different on the first day ($P \leq 0.05$). Accuracy results based on the RSD, obtained on different days, were considered acceptable, being within the limit for validation of chromatographic methods (ANVISA 2003; Tamim and Doerr 2003; Ribani et al. 2004). No significant differences ($P>0.05$) were observed in retention times for the 3 days in all amine standards.

The recovery was better in solutions prepared with high amine standard concentrations (Table 4). These results differ from Baston et al. (2008); they used three different concentrations (0.5, 1.0, and 2.0 mgL^{-1}) and described an excellent recovery for spermine (>99 %) and cadaverine and spermidine (>95 %), and relatively low for putrescine (>94 %) and tyramine (>92 %) at all concentrations. These authors also indicated that recoveries exceeding 100 % are normal, but it should not exceed 105 %, which indicates an equipment problem (column and detector). The variations in recovery may be related to changes in the methodology, filters with smaller diameter pores, derivatization substances, different concentrations, gradient systems, and other factors. Vinci and Antonelli (2002) reported that parameters such as solvent, extraction, pH, and conditions chosen for derivatization could

Table 4 Recovery, limit of detection, and limit of quantification for the proposed chromatographic method

Biogenic amines	Recovery (%)			LOD (μgL^{-1})	LOQ (μgL^{-1})
	Mix A	Mix B	Mix C		
Tyramine	84.30	91.58	112.22	0.03	0.15
Putrescine	90.42	94.59	94.59	0.25	0.80
Cadaverine	73.43	106.54	111.16	1.25	5.00
Spermidine	81.33	91.27	99.43	0.25	0.78
Spermine	64.40	90.65	95.89	0.06	0.50

Mix A=100 mgL^{-1} for each amine; mix B=150 mgL^{-1} for each amine; mix C=200 mgL^{-1} for each amine

LOD limit of detection, *LOQ* limit of quantification

Table 5 Results of robustness for variations in mobile phase concentration, wavelength, flow rate, and derivatization time

Parameters	Biogenic amines (mgL^{-1})				
	Tyramine	Putrescine	Cadaverine	Spermidine	Spermine
Acetonitrile/water (*v:v*)					
41:59	407. 20±6.97 a	180.98±5.79 a	191.23±9.18 a	165.86±2.09 a	169.36±6.67 a
42:58	411.29±10.50a	173.71±4.80 b	201.16±2.56 a	168.17±2.42 a	170.28±3.78 a
43:57	412.56±7.88 a	174.69±1.06 b	192.92±7.73 a	165.02±4.91 a	172.23±7.50 a
Detector (nm)					
197	410.97±6.33 a	176.37±3.49 a	199.96±1.49 b	176.86±4.36 a	154.02±2.36 b
198	410.75±5.47 a	179.02±5.21 a	210.40±5.63 a	178.43±3.45 a	167.35±2.49 a
199	412.05±4.14 a	173.73±6.56 a	212.62±3.43 a	172.53±3.73 a	172.69±6.11 a
Flow rate ($mL\,min^{-1}$)					
0.95	415.81±6.66 a	180.31±4.58 a	196.98±4.97 a	179.99±3.61 a	169.22±0.70 a
1.00	402.29±7.26 ab	184.24±3.65 a	198.68±4.92 a	176.92±3.85 a	169.32±1.44 a
1.05	397.38±9.85 b	173.46±1.64 b	197.73±1.95 a	183.16±1.36 a	169.51±1.73 a
Time of derivatization (min)					
15	402.31±5.43 a	159.63±3.62 b	174.70±5.81 b	184.06±2.82 b	172.18±4.91 b
20	409.68±5.48 a	181.42±6.03 a	195.94±6.08 a	191.99±5.47 ab	180.41±1.12 a
25	408.56±3.87 a	184.10±2.05 a	194.82±3.18 a	192.78±2.30 a	186.01±1.36 a

Results expressed as mean±SD. Different letters in rows represent significantly different averages ($P \leq 0.05$)

influence recovery values, but, in this case, the same methodology was used for all determinations.

LOD and LOQ values were determined by the injection of serial dilutions from the lowest amine concentration used in the linearity. Tyramine and cadaverine presented the lowest and highest values, respectively, for LOD and LOQ (Table 4). Baston et al. (2008) found values between 5 and 30 μgL^{-1} for LOD and 10 to 60 μgL^{-1} for LOQ for the same amines; however, their protocol used a signal-to-noise ratio and wavelength of 254 nm for the determination of limits. The most important factors that determined these results were the higher sensitivity of the detector wavelength (198 nm) used in the HPLC system and the derivatized substance (benzoyl chloride), which, in addition to the advantage of rapid derivatization, has a better stability than other derivatizing agents, such as *o*-phthalaldehyde or dansyl (Özdestan and Üren 2009).

The results of robustness in different parameters are shown in Table 5. Changes in mobile phase concentration did not modify the biogenic amine determination. The values for putrescine are significantly different ($P \leq 0.05$) using the mobile phase concentration of 41:59. For wavelength variations, cadaverine and spermine showed significant differences ($P \leq 0.05$) at 197 nm. Baston et al. (2008) found little difference in the results using two wavelength variations (249 and 259 nm).

Another parameter evaluated was flow rate. This variable was associated with significant differences ($P \leq 0.05$) for putrescine and tyramine at 1.05 and 0.95 $mL\,min^{-1}$, respectively. For derivatization time, 15 min was significantly different ($P \leq 0.05$) for all standard amines, indicating that 15 min is insufficient and that 20 min or more is required for appropriate derivatization.

Table 6 Biogenic amine results from chicken breast meat stored in two commercial packages

Biogenic amines	Package A			Package B		
	Mean±SD	Range	RT	Mean±SD	Range	RT
Tyramine	230.72±78.07a	68.41–367.79	2.35	195.42±40.26 b	108.47–259.06	2.40
Putrescine	6.93±2.59 a	1.76–11.09	4.42	6.73±3.20 a	3.35–33.57	4.38
Cadaverine	4.71±2.01 a	1.37–8.96	5.28	4.57±2.31 a	1.18–9.73	5.30
Spermidine	9.80±3.08 a	6.40–15.94	7.10	10.77±3.31 a	6.33–18.84	7.17
Spermine	16.03±4.02 a	9.67–27.77	11.81	18.98±5.26 a	12.56–38.00	11.75

Different letters in rows represent significantly different averages ($P \leq 0.05$). Package A: chicken meat in expanded polystyrene tray; package B: chicken meat in plastic bag

RT retention time

All validation parameters showed a satisfactory response. When compared with other researches in chicken meat and chicken meat products, the proposed method has the advantage of short running time, high resolution, and stable and reproducible isocratic separation of all biogenic amines. Baston et al. (2008) showed a precise, sensible, selective, and reproducible method to quantify nine biogenic amines; nevertheless, it was a complex extraction and 30 min of running time. Silva and Gloria (2002) identified six amines used as gradient separation with 80 min to running time. Vinci and Antonelli (2002) used a method with high sensitivity, good precision and recoveries but the running time took 25 min.

The biogenic amine values in commercial chicken meat samples was determined used the proposed method. Tyramine had the highest value in chicken breast meat from the two types of commercial packaging (Table 6). Both presentations were significantly different ($P \leq 0.05$) for tyramine; no differences ($P > 0.05$) were observed for the other amines. These results are consistent with those described by Rokka et al. (2004), who found 100 $mgkg^{-1}$ of tyramine, 82 $mgkg^{-1}$ for spermine, and less than 15 $mgkg^{-1}$ for putrescine, cadaverine, and spermidine in chicken meat stored under refrigeration for 12 days.

Vinci and Antonelli (2002) compared amine levels produced by beef and chicken meat and observed that chicken meat conservation was critical because nonphysiological biogenic amines increased earlier and more rapidly than in beef. They attribute this result to differences in chicken muscles, where there are shorter fibers that can be easily attacked by proteolytic enzymes, increasing the amount of amino acid precursors responsible for amine biosynthesis.

Muscle cells rupture and, consequently, amino acid release can be attributed to freezing temperatures. Ruiz-Capillas and Jiménez-Colmenero (2004) proposed that the freezing process can lead to structural and chemical changes in meat and that these modifications depend on chilling treatments and storage conditions. Kozová et al. (2009) found values of 30–70 mgL^{-1} for spermidine and 250–300 mgL^{-1} for spermine in chicken meat frozen for 6 months. According to Silva and Gloria (2002), spermine values can be reduced during storage time under refrigeration, where some microorganisms use this polyamine as a source of nitrogen.

Other researchers present results with significant variations. Sander et al. (1996) found high levels of cadaverine (541.19 $mgkg^{-1}$), putrescine (244.19 $mgkg^{-1}$), and tyramine (220.39 $mgkg^{-1}$), and low levels of spermine (4.08 $mgkg^{-1}$) and spermidine (5.72 $mgkg^{-1}$) in fresh chicken carcasses. Balamatsia et al. (2006) described levels of tyramine and cadaverine at less than 10 $mgkg^{-1}$ and initial values of putrescine exceeding 48 $mgkg^{-1}$ and spermine at 53 $mgkg^{-1}$. Gallas et al. (2010) reported concentrations of spermine (17.9 mgL^{-1}), putrescine (26.4 mgL^{-1}), cadaverine (8.5 mgL^{-1}), and spermidine (7.3 mgL^{-1}) after 3 days of storage under refrigeration (4±2 °C).

Conclusion

The proposed method allows for the simultaneous identification and quantification of five biogenic amines by HPLC in chicken meat. The advantages of this method were easy extraction, effective derivatization, and high resolution in a short assay using an isocratic separation with acetonitrile and water. This study demonstrated good selectivity, linearity, accuracy, recovery, robustness, LOD, and LOQ. The method was successfully applied to commercial chicken meats, showing that tyramine was at the highest levels in both commercial packages.

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6.2 PAPER II

Biochemical changes in alternative poultry meat during refrigerated storage

Alterações bioquímicas em carne de aves alternativas durante o armazenamento em refrigeração

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Abstract

The aim of this study was evaluated the physical and chemical changes in five types of alternative poultry meat keep at refrigerated conditions (4±1°C) during 18 days. For this purpose, breast meat of conventional, organic and free-range chicken, duck and quail were purchase from markets of Niteroi city (Rio de Janeiro, Brazil). Levels of pH, thiobarbituric acid reactive substances (TBARS) and biogenic amines (cadaverine and putrescine) were determinate. Statistic evaluation was performed using analyze of variance and Tukey test. The results showed a gradual and proportional increment of pH values (between 5.5 and 6.5) in all poultry meats during the storage time; an increment of TBARS values in the first days of storage, remaining stables to the end of experiment. A significant increment of putrescine values was observed after the sixth day remaining stable in conventional chicken and quail until the end of experiment while significant reduction was observed in the rest of poultry meats. Finally, only conventional and organic chicken and quail showed a gradual increment during storage time. In conclusion, significant biochemical changes was observed during the storage time being that pH, cadaverine and putrescine values could be parameters using like deterioration indicators for these products.

Keywords: biogenic amines, chicken, duck, pH, quail, quality, TBARS.

Resumo

O objetivo do presente estudo foi avaliar as mudanças físico-químicas de cinco tipos de carnes de aves alternativas mantidas em condições de refrigeração (4±1°C) durante 18 dias. Para essa finalidade utilizou-se carne de peito de frangos convencional, caipira e orgânico, pato e codorna adquiridos de supermercados na cidade de Niterói (Rio de Janeiro, Brasil) e determinaram-se os níveis de pH, substâncias reativas ao ácido tiobarbitúrico (SRATB) e aminas biogênicas (cadaverina e putrescina). Os resultados indicaram um incremento gradativo e proporcional dos valores de pH (entre 5,5 e 6,5) nas carnes de aves durante o tempo de estocagem; um aumento dos valores de SRATB nos primeiros dias de estocagem, mantendo-se invariáveis até o final do experimento. Em todas as aves foi observado um incremento significativo dos valores de putrescina após o sexto dia, mantendo-se estáveis no frango convencional e a codorna até o final do experimento, enquanto o resto das carnes diminuiu significativamente. Finalmente, foi observado um incremento gradativo apenas na codorna e nos frangos convencional e orgânico. Conclui-se que houve mudanças bioquímicas significativas nas carnes avaliadas durante o tempo de estocagem, sendo que os valores de pH, cadaverina e putrescina seriam parâmetros que podem ser utilizados como indicadores de deterioração destes produtos.

Palavras-chave: aminas biogênicas, codorna, frango, pato, pH, qualidade, TBARS.

Introduction

The Brazilian poultry industry sector of the contemporaneous economy has undergone profound changes over the past 40 years, presenting a set of changes related to the organizational structure encompassing much of the production processes, administrative and work organization (Buzanello and Moro, 2012). Due to the increase global consumption of alternative meats, the poultry industry constantly searches different options to attend consumer demands. In Brazil, the alternative poultry production system was initially implemented by small and medium producers as an opportunity to offer differentiated products of higher quality providing producers higher income through added value in relation to intensive production system. However, in recent years the alternative production is widely implemented in the poultry industry (Santos et al., 2012).

The Brazilian free-range chicken system is regulated by the Brazilian legislation; according to that, the chicken named free-range is fed exclusively with plant origin ration and the use of

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growth promoters is prohibited. Also, is recommended that the birds are reared in extensive and three square meters of pasture per animal and the slaughter takes place at the minimum age of 85 days (Brasil, 1999).

An increasing number of consumers demanding healthier and naturally grown foods have favored organic live-stock farming, which is reputed to be environmentally friendly, allied with promotion of animals good health, with high welfare standards results in high quality products (Castellini et al., 2002)

The aim of this study was determinate the biochemical changes in alternative poultry meats and evaluated if these biochemical parameters could be used as indicators of poultry meat deterioration.

Materials and methods

The present study evaluated the changes of pH, thiobarbituric acid reactive substances and biogenic amines (cadaverine and putrescine) in five types of poultry meat keep at refrigerated (4±1°C) conditions for 18 days.

Ten breast meat (m. *Pectoralis maior*) from conventional, ten from free-range and ten from organic chicken (*Gallus gallus domesticus*) were used. Furthermore, ten breast from duck (*Anas platyrhynchos domesticus*) and twenty from quail (*Coturnix coturnix*) were used. All samples were purchased from local markets in Niteroi city (Rio de Janeiro, Brazil). Frozen samples were thawed overnight at 4±1°C and were aseptically cut into pieces of 100g, placed in plastic bags for refrigerated storage at 4±1°C for 18 days. In case of quail an entire breast of each carcass was used. Biochemical changes were evaluated every two days during 18 days on refrigeration storage.

All reagents used in the present study were of analytical grade. Cadaverine and putrescine standards (Sigma Aldrich, St. Louis, MO, USA) and 2-thiobarbituric acid (4,6-dihydroxy-2-mercaptopyrimidine) (Spectrum Chemical Mfg. Corp. New Jersey, USA) were purchased. Stock solution with 40 µg of cadaverine and putrescine were prepared in 0.1 *N* HCl and stored at 4±1°C. Different dilutions were performed and used for the calibration curve.

The progress of oxidation was determined by the thiobarbituric acid reactive substances (TBARS) test according to Tarladgis et al. (1960). In brief, 10g of sample were manually minced, homogenized with 97.5mL of distilled water and transferred to a distillation tube which was added 2.5mL of HCl (4*N*). This sample was then distilled and the first 50mL of distillate was collected. Next, 5mL of the distillate were added to 5mL of 0.02M thiobarbituric acid and were heated in a boiling water bath (100°C) for 35 min for accelerate the reaction and, consequently, color development. The samples were immediately cooled with water and the absorbance was measured at 528 nm on a Smartspec Plus spectrophotometer (BioRad, Hercules, CA, USA). The final values were expressed as milligram of malondialdehyde (MDA) per kilogram. The pH values were performed by using a digital pH meter (Digimed® DM-22) equipped with a electrode (Digimed® DME-R12) after briefly homogenization of 10 g of muscle sample with 90mL of distilled water (Conte-Júnior et al., 2008).

Biogenic amines determination was performed in accordance with a modified procedure proposed by Conte Junior et al. (2006). Extraction were carried out using 5g of meat and 5mL of 5% perchloryc acid; the mixture was homogenized (Certomat® MV, B. Braun Biotech International) for 30 seconds in ten minutes intervals up to one hour; the sample tubes were kept under refrigeration (4±2°C) during the whole process. The homogenate was centrifuged at 503*g* for 10min at 4±1°C (Hermle Z 360 K) and filtered with Whatman No. 1 paper. The filtrates were neutralized (pH>6) with 2*N* NaOH and kept in ice bath (0±1°C) for 20 min. A second filtration and alkalynization with NaOH (pH>12) were performed in the same conditions.

The derivatization was performance with addition of 40mL benzoyl chloride, followed by homogenization in vortex for 15 sec and kept at room temperature (25±2°C) for 20 min. The biogenic amines were collected through liquid partitioning with 1000 mL of diethyl ether, which was proceeded in two times. The ether layers containing amines were evaporated to dryness under nitrogen stream (Sample Concentrator Techne, Cambridge, UK). Finally, the residue was dissolved in 1000mL of mobile phase (acetonitrile:water) and stored at 4±1°C.

The chromatographic system consisted of a Shimadzu LC/10AS liquid chromatography coupled to SPD/10AV UV-Vis detector and C-R6A Chromatopack integrator. Amines separation were performed on Teknokroma Tracer Extrasil ODS2 (15 x 0.46cm id., 5µm) column equipped with a Supelco, Ascentis C18 (2 x 0.40 cm, id. 5µm) guard column, in isocratic condition. The mobile phase was prepared by mixing acetonitrile (Tedia®) with ultrapure water purified on Millipore Simplicity (Millipore, Molsheim, France) at 42:58 (v/v); and degassed in ultrasonic bath (Cleaner USC 2800 A). The chromatography conditions were: flow rate of 1 $mL.min^{-1}$, injection volume of 20µL, column temperature of 20°C and detector wavelength set at 198 nm. Injection was performed using a 50µL syringe (Hamilton TM 705) and total run time of 15 minutes. Injection of pure acetonitrile for 10 min was used between each sample for conditioning the HPLC system. Presence of biogenic amines were identified by retention time and quantified by peak area.

A two-way analysis of variance with repeated measures on two factors was used to identify differences in pH, TBARS and biogenic amines content in different poultry meat. When a significant F was found, additional post hoc tests with Tukey adjustment were performed. For the interpretation, data set was divided, for all above-mentioned analyses, in four periods: Period 0 (P0) – analysis of day 0 of storage; Period 1 (P1) – analysis of the first six days of storage; Period 2 (P2) – analysis of the subsequent six days; and Period 3 (P3) – analysis of the last six days of storage. Statistical significance was set at the 0.05 level of confidence. All analyses were performed using a commercially available statistical package Graphpad (2007) Prism version. 5.00 for Windows (GraphPad Software, San Diego California USA).

Results and discussion

At the beginning of the experiment quail, duck, free-range and conventional chicken had similar values between 5.57 and 5.77. On the other hand, organic chicken showed values of pH above of 6.0. Through all of storage period all meats showed a significant increase demonstrating that pH can be considered a quality parameter (Figure 1). These results are similar to those found by Castellini et al. (2002) that determined pH values of 5.75 and 5.80 for organic chickens slaughtered at 56 and 81 days of age respectively; these values would be related to the rearing system of those birds in open environments and better

welfare conditions which would reduce the stress pre-slaughter and further consumption of muscle glycogen. Free-range chicken had acidic values which are consistent with the results of Cheng et al. (2008) and Faria et al. (2009) who observed pH values between 5.70 and 5.90 after 24 h *post mortem* in free-range chicken, explaining that larger diameter muscle fibers was related to slow-growing chicken, which have higher glycolytic activity and reduced glycogen reserves before slaughter, fact that determine the final pH observed.

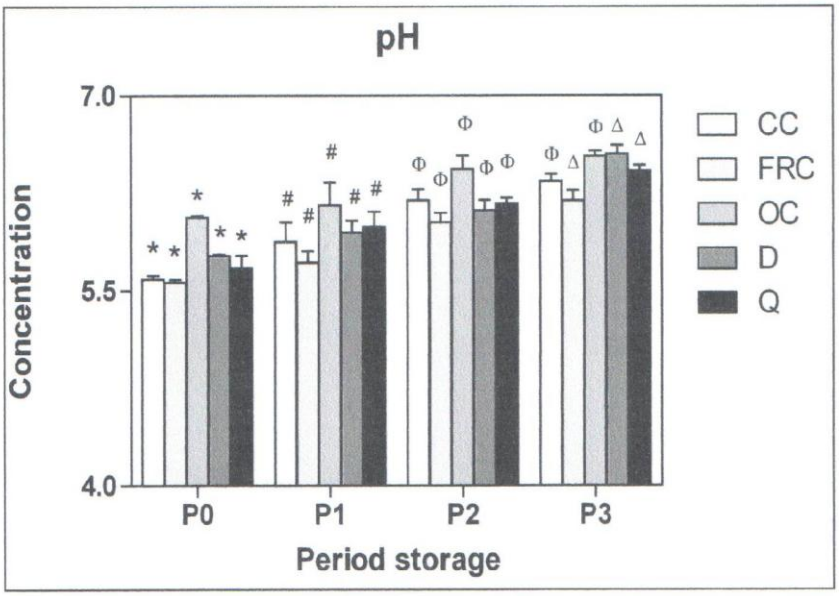

Figure 1: pH values in different poultry meats in refrigerated conditions (4 ± 1 °C) storage during 18 days. Different symbols over the bars indicate significant differences between of storage periods in the same kind of poultry meat. Abbreviations: CC = Conventional chicken, FRC = Free-range chicken, OC = Organic chicken, D = Duck, Q = Quail, P0 = Day 0 of storage; P1 = 1 – 6 days of storage, P2 = 7 - 12 days of storage, and P3 = 13 -18 days of storage

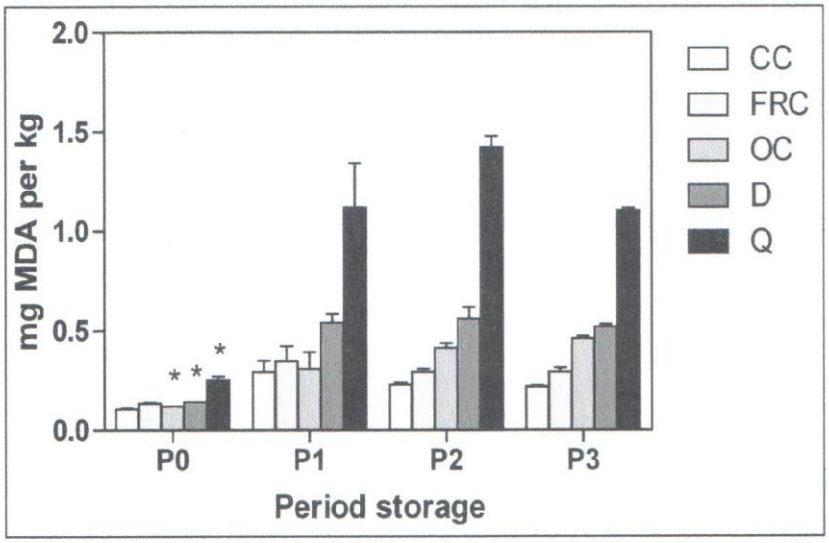

Figure 2: Thiobarbituric acid reactive substances (TBARS) concentration in different poultry meats in refrigerated conditions (4 ± 1 °C) storage during 18 days. The symbol * over the bars indicate significant differences between of periods storage in the same kind of poultry meat. Abbreviations: CC = Conventional chicken, FRC = Free-range chicken, OC = Organic chicken, D = Duck, Q = Quail, P0 = Day 0 of storage; P1 = 1 – 6 days of storage, P2 = 7 - 12 days of storage, and P3 = 13 -18 days of storage

All poultry meats showed lower initial levels of TBARS (0.10–0.25mg.kg^{-1}). Conventional and free-range chicken meat showed no difference during storage time (Figure 2). On the other hands, organic chicken, duck and quail showed a significant increase in the first six days of storage and then these values remained stable until the end of the storage period; this fact suggest that a early increment of MDA in these birds indicate an early stage of rancidity, which would not be suitable as quality indicator like pH values. The quail showed highest values (above of 1mg.kg^{-1}) compared to other birds; these results can be explained because the quail were purchased with the skin that may have contributed to the increase of oxidative rancidity, while all chicken breasts were acquired without skin.

Our results were similar to reported by Alasnier et al. (2000) who determined low initial levels of lipid oxidation 0.03 mg.kg^{-1} followed by a linear increment until 0.30 mg.kg^{-1} at day 14 in chicken breast meat; these authors suggested that lower ratios of vitamin E in chicken meat prevents the oxidation of long-chain PUFAs in the initials days and after the antioxidant reserves exhausted, the lipid oxidation is more evident. On the other hands, Castellini et al. (2002) and Castellini et al. (2006) evaluating the physical and chemical characteristics of organic chickens and found TBARS values above 2 mg.kg^{-1}, 24 h *post mortem*. They explained that the type of rearing of these birds, in open environments with intense muscle activity, could increase muscle oxidative metabolism and the production of free radicals. Husak et al. (2008) evaluated organic, free-range and conventional chickens, found TBARS values between 0.12 and 0.19 mg.kg^{-1}, not considered indicative of rancidity in any chicken.

Putrescine levels showed a gradually increase in conventional chicken and quail and after the first six days of storage it increased at significant levels. Similar behavior was observed in free-range chicken but the highest levels were detected between 7 and 12 day of storage, followed by a significant decrease in the last period of experiment. Organic chicken showed a slightly increment between 7 and 12 day of storage but it was not significant. Duck levels also started at low level and significantly increased between 2 and 12 day of storage; then it was observed a slightly decrease (Figure 3).

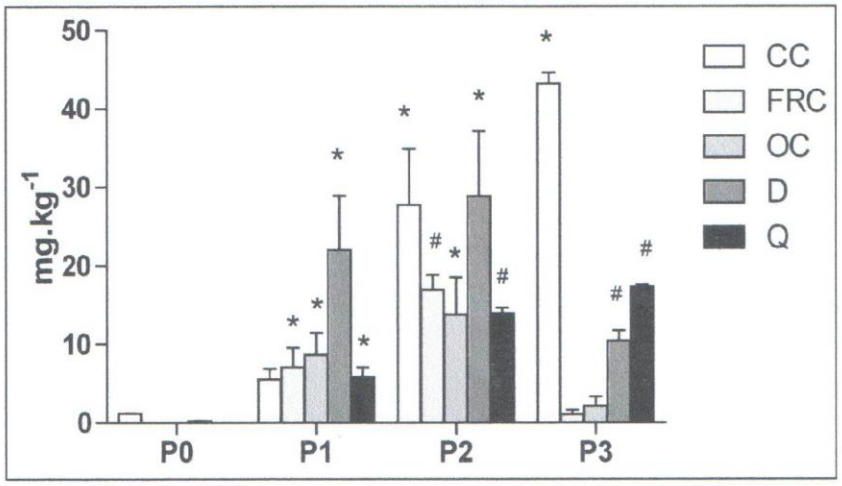

Figure 3: Putrescine concentration in different poultry meats in refrigerated conditions (4 ± 1 °C) storage during 18 days. The symbols (*,#) over the bars indicate significant differences between of periods storage in the same kind of poultry meat. Abbreviations: CC = Conventional chicken, FRC = Free-range chicken, OC = Organic chicken, D = Duck, Q = Quail, P0 = Day 0 of storage; P1 = 1 – 6 days of storage, P2 = 7 - 12 days of storage, and P3 = 13 -18 days of storage

Regarding, cadaverine it was observed that only organic meat showed detectable values at day 0. Conventional chicken showed the lowest values until the 12 day of storage when significantly increased. Free-range progressively increased until 12 day, after that a significantly decrease was observed. Organic chickens and quail showed a gradually and significantly increase in all periods of storage. Duck values slightly increased in the first and then remain stables to the end of period storage (Figure 4).

Putrescine and cadaverine values were rather low in comparison to the levels reported by Sander et al. (1996), who found 200 and 500 mg.kg^{-1} respectively in chicken carcass 24 h *post mortem*. On the other hand, different authors described a gradually increase for those amines during storage. Silva and Gloria (2002) found 20.4 and 4.3 mg kg^{-1} for putrescine and cadaverine respectively in chicken meat storage at 4°C for 15 days; similar results were obtained by Rokka et al. (2004) for both amines in chicken meat storage at different temperatures between 2 and 8 °C; finally Balamatsia et al. (2007) who showed a linear increase from 53.8 to 409.6 mg.kg^{-1} and 19.8 to 252.8 mg.kg^{-1} for putrescine and cadaverine, respectively, in chicken meat storage at 4°C for 17 days. On the other hand, ours results of putrescine for duck meat were different to Dadáková et al. (2012) who determined values of 3.2 mg.kg^{-1} which slightly declined in the first week.

Vinci and Antonelli (2002) comparing biogenic amines, including putrescine and cadaverine, in beef and chicken stored at 4±1°C, reported that amines in white meat increases earlier than in red meat; they explained that it is due to shorter fibers in chicken than beef, which can be easily attacked by proteolytic enzymes, resulting in the increased availability of amino acid precursors of biogenic amines. Tamim and Doerr (2003) determinated that levels of putrescine depends on the presence of ornithine, which in turn is produced from arginine degradation; consequently their formation may be ruled by arginine-utilizing microorganisms that produce ornithine as the substrate of decarboxylation. The putrescine levels reduction in duck, free-range and organic

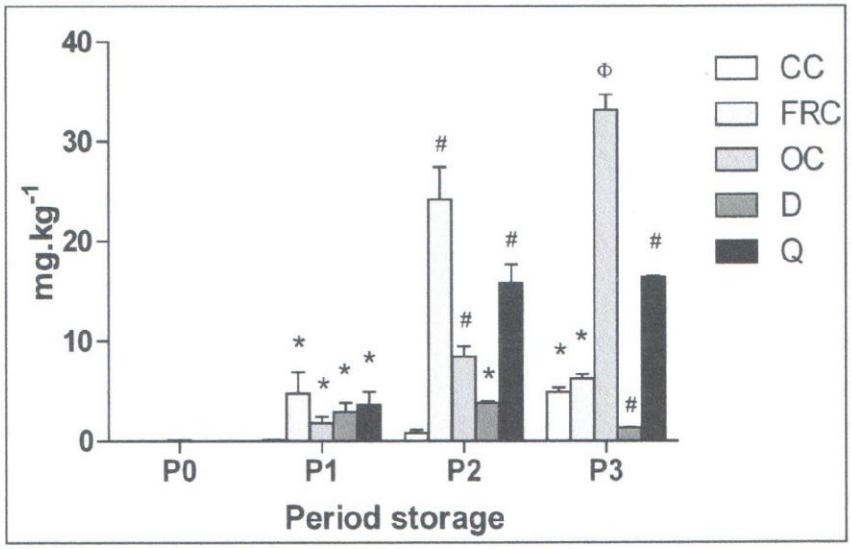

Figure 4: Cadaverine concentration in different poultry meats in refrigerated conditions (4 ± 1 °C) storage during 18 days. The symbols *, ϕ, # over the bars indicate significant differences between of periods storage in the same kind of poultry meat. Abbreviations: CC = Conventional chicken, FRC = Free-range chicken, OC = Organic chicken, D = Duck, Q = Quail, P0 = Day 0 of storage; P1 = 1 – 6 days of storage, P2 = 7 - 12 days of storage, and P3 = 13 -18 days of storage

chicken in the latter period of storage can be related to the small amount of arginine. This condition is principally related to feeding and management. Khajali and Wideman (2010) indicated that reserves of arginine are consumed during stressful conditions in conventional chickens. The requirements of this amino acid and standard management are well-known in conventional chicken but a little information is available in other birds. The same assumption can be used with the requirements of lysine and cadaverine production.

The bacterial spoilage is another factor related with the putrescine and cadaverine production in meat products. In this case, although bacterial load was not evaluated, has been reported that variations in cadaverine levels could be due to differences in the capacity of the microorganisms, species and strain to produce cadaverine (Delgado-Pando et al., 2012).

Our results are important because these biochemical parameters could be useful and easily implemented by poultry industry as a possible indicator of poultry meat quality. According to Balamatsia et al. (2007), the changes in levels of amines were suitable as potential indices of fresh chicken meat quality. However, the authors indicated that these results corresponded to samples from one poultry plant and their general application must be verified.

Conclusion

The evaluation of five poultry meat showed biochemical changes during the storage time. Values of pH and putrescine in all poultry and cadaverine in quail, organic and free-range chicken, had a significant increase and could serve as indicators of storage time after 12 days. TBARS did not seem to have relationship with the storage time because increase only took place in the first days. Further studies should be carried out to evaluated others biochemical indicators (e.g.: spemine, spermidine, histamine, tyramine) and the correlation with microbiological parameters.

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