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SABRINA DA COSTA SILVA ANDRADE

PARÂMETROS DE QUALIDADE PARA AVALIAÇÃO DO FRESCOR DE TAINHA (*Mugil platanus*) E CORVINA (*Micropogonias furnieri*) E INFLUÊNCIA DA TEMPERATURA DE ESTOCAGEM NA VALIDADE COMERCIAL DESTAS ESPÉCIES

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RESUMO

A tainha (*Mugil platanus*) e a corvina (*Micropogonias furnieri*) são peixes de elevado interesse comercial em alguns países como o Brasil. No entanto, flutuações na temperatura de estocagem constituem uma das principais causas para perda da qualidade do peixe em todo mundo. Especificamente no Brasil, os parâmetros oficiais em vigor para avaliação da qualidade do peixeainda são pouco eficientes sob o ponto de vista analítico, por não detectarem as alterações iniciais que ocorrem no processo de deterioração, o que dificulta um julgamento preciso e criterioso do estado de frescor deste alimento. Desta forma, o objetivo do presente estudo foi estabelecer parâmetros de qualidade que possam refletir de forma mais efetiva o frescor de peixes marinhos, utilizando-se como modelos biológicosa tainha e corvina, simulando uma variação de 5°C na temperatura de estocagem (0±1 e 5±1ºC) para estimar a validade comercial destas espécies. Tendo em vista que a avaliação sensorial é um método rápido para inferir sobre a qualidade do pescado, um protocolo sensorialfoi desenvolvidopara avaliação da tainha. Para a corvina, esta análise não foi necessária devido à existência de um protocolo elaborado em estudos prévios. O índice de qualidade ao redor de 16 foi proposto pelos julgadores como limite de aceitabilidade e, com base neste valor, a tainha mantida a 0±1ºC e 5±1ºC foi rejeitada no 14º e 7º dias de estocagem, respectivamente, entretanto a carga microbiana de 7 log UFC.g^{-1} para bactérias mesófilas estabelecida como limite máximo aceitável pela ICMSF (1986) foi atingida no 20º e 14º dias de estocagem, respectivamente. Com relação às alterações químicas, o teor de adenosina monofosfato (AMP), inosina monosfato (IMP), inosina (HxR) e Hx (hipoxantina) e o nível de histamina (HI), putrescina (PU), cadaverina (CA) e tiramina (TI) foram determinados por HPLC-UV e HPLC-FLD, respectivamente,durante estocagem da corvina e tainha Em ambas as espécies, o teor de IMP declinou e o nível de Hx aumentou, sendo que apenas na tainhahouve aumento significativo ($p<0,05$) do teor de HxR até o tempo T3 (10º ao 14º dia de estocagem)que posteriormente declinou.Na corvina, o nível de HI e TI aumentou significativamente ($p<0.05$) principalmente nas amostras mantidas a 5±1ºC, enquanto quena tainha ocorreu aumento significativo ($p<0.05$) apenasdos teores de PU e TI. A temperatura de estocagem também influenciouasalterações bacteriológicas em ambas as espécies. A fase de latência das bactérias mesófilas e psicrotróficas nas amostras estocadas a 0±1ºC foi maior que o observado nas amostras estocadas a 5±1ºC, havendo multiplicação acelerada desses grupos bacterianos na temperatura mais elevada, o que determinou na diminuição da validade comercial da tainha e corvina em seis e dez dias, respectivamente. Conclui-se que o protocolo sensorial elaborado auxiliouna avaliação da qualidade da tainhacom parâmetros adequados para estimar a validade comercial desta espécie, entretanto a rejeição precisa do produto deve ser feita com base nas análises físico-químicas e microbiológicas; variações na temperatura de estocagem induziram expressivamente a validade comercial dos peixes; IMP, Hx, HI e TI foram considerados parâmetros químicos adequados para avaliação da qualidade da corvina, enquanto que para avaliação da tainha, os parâmetros IMP, HxR, Hx, PU e TI foram os que obtiveram maior correlação com a perda de qualidade; a técnica de cromatografia líquida de alta eficiência utilizada permitiu a adequada separação e identificação dos compostos analisados.

Palavras-chave: tainha, corvina, aminas biogênicas, nucleotídeos, temperatura de estocagem, índices de qualidade.

ABSTRACT

Mullet (*Mugil platanus*) and whitemouth croaker (*Micropogonias furnieri*) are fish of high commercial interest in some countries like Brazil. However, fluctuations in the storage temperature are one of the main causes for loss of quality fish in the world. Specifically in Brazil, the official parameters in place for evaluation the fish quality are still inefficient in the point of view analytical it did not detect the early changes that occur in the deterioration process, which makes a precise and careful judgment of the freshness of this food. Thus, the aim of this study was to establish quality parameters that may reflect more effectively the freshness of marine fishes, using as biological models the mullet and whitemouth croaker, simulating a range of 5ºC in the temperature storage (0±1 and 5±1°C) to estimate the shelf life these species. As sensory evaluation is a quick method to infer the quality of the fish, a sensory protocol was developed to evaluate the mullet. For whitemouth croaker, this analysis was not necessary because a protocol was developed in previous studies. The quality index around 16 was proposed by panelists as the limit of acceptability and, based on this value, the mullet stored at 0±1ºC and 5±1ºC was rejected in 14 and 7 days of storage, respectively, however the microbial load 7 log CFU.g^{-1} for mesophilic established as maximum acceptable limit by ICMSF (1986) was achieved in 20 and 14 days of storage, respectively. In relation to chemical changes, the level of adenosine monophosphate (AMP), inosine monosphate (IMP), inosine (HxR) and hypoxanthine (Hx) and the level of histamine (HI), putrescine (PU), cadaverine (CA) and tyramine (TI) were determined by HPLC-UV and HPLC-FLD, respectively, during storage of whitemouth croaker and mullet.In both species, the concentration of IMP declined and the level of Hx increased. Only in the mullet,the content of HxR increased significantly ($p<0.05$) until time T3 (10th to 14th day of storage) and subsequently declined. In the whitemouthcroaker, the level of HI and TI increased significantly ($p< 0.05$), especially in samples stored at 5±1ºC, while in the mullet,only the levels of PU and TI increased significantly ($p< 0.05$). Storage temperature also influenced the microbiological changes in both species. The lag phase of mesophilic and psychrotrophic bacteria in samples stored at 0±1ºC was greater than that observed in samples storage at 5±1ºC, with accelerated multiplication of bacterial groups at higher temperature, which resulted in decrease in the shelf life of the mullet and whitemouthcroakerin six and ten days, respectively. We conclude that the sensory protocol helped to evaluate the quality of mullet with adequate parameters to estimate the shelf life of this specie, however the precise rejection of the product must be based on the physicochemical and microbiological analyzes; variations in storage temperature significantly induced the shelf life of the fish; IMP, Hx, HI and TI were considered suitable chemical parameters for assessment the quality of whitemouthcroaker, while for evaluation of mullet, the IMP, HxR, Hx, PU and TIparameters obtained the largest correlation with loss of quality; the high performanceliquid chromatography technique provided adequate separation and identification of the compounds analyzed .

Keywords: mullet, whitemouthcroaker, biogenic amines, nucleotides, storage temperature, quality index.

SUMÁRIO

1 INTRODUÇÃO

A procura por alimentos nutritivos, aparência agradável, baixo custoe com garantia de inocuidadevem aumentando nas últimas décadas. Neste cenário, destaca-se o aumento do consumo do peixe, tanto pelos aspectos relacionados a saúde, com benefícios indiscutíveisem função do perfil proteicocom aminoácidos de alto valor biológico, e ácidos graxos poli-insaturados, especialmente os da série ômega 3 (ω-3) quanto pela influência da gastronomia japonesa, diretamente correlacionada a vida saudável e longevidade. No entanto, em função de sua composição química, atividade de água elevada, teor de gorduras insaturadas facilmente oxidáveis e, principalmente, ao pH próximo à neutralidade, este alimento é altamente susceptível à deterioração, situação que justifica um cuidado mais efetivo e criterioso para garantir a manutenção da qualidade.

Desta forma, para que as perdas nutricionais e produtivas sejam minimizadas e os riscos de contaminação controlados, é imperativo ações efetivas de manutenção da qualidade em toda cadeia produtiva, o que inclui artes de pesca, embarcações, área geográfica da pesca, até as etapas após captura e processamento. O estresse causado ao peixe durante a captura, a manipulação inadequada nas etapas do processamento, e desvios na manutençãoda cadeia de frio podem acelerar as alterações autolíticas *post mortem* e a deterioração microbiana, diminuindo a validade comercial desta matriz alimentar.

A avaliação da qualidade do pescado pode ser realizada pela observação das alterações sensoriais ocorridas no decorrer do período de estocagem, assim como por análises físico-químicas e bacteriológicas, entretanto, os parâmetros oficiais em vigor no Brasil ainda são falhos por não detectarem as alterações iniciais que ocorrem no processo de deterioração, o que dificulta a avaliação precisa e criteriosa do estado de frescor deste alimento. Metabólitos importantes formados na etapa inicial do processo de deterioração do pescado como os produtos da degradação dos nucleotídeos, assim como a presença de aminas biogênicas podem constituir ferramentas analíticas são efetivas para avaliação da qualidade desta matriz alimentar. Outro fator relevante é que as alterações que ocorrem no pescado pós-captura dependem de variados fatores tais como a espécie envolvida, o tamanho da espécie, o tipo de flora contaminante,o teor de gordura do pescado, o tipo de

alimentação, bem como o local e a época de captura e os parâmetros oficiais, em sua maioria, são estabelecidos de forma geral para todas as espécies. Desta forma, o objetivo geral do presente estudo foi estabelecer parâmetros de qualidade que possam ser utilizados para avaliação do frescor deespécies de peixe de elevado interesse comercial no litoral fluminense, tais como a tainha (*Mugil platanus*) e a corvina (*Micropogonias furnieri*), e avaliar a influência do abuso de temperatura na estocagem por parâmetros analíticos quantitativos, bem como estimar o “gap” na validade comercial destas espécies.

2 REVISÃO DE LITERATURA

A seguir serão abordados aspectos relevantes que auxiliam no entendimento do assunto proposto tais como a pesca no Brasil e Estado do Rio de Janeiro, o peixe como alimento e o controle da qualidade do peixe fresco, sendo abordados neste último tópico os fatores que afetam a qualidade do peixe, os parâmetros de qualidade oficiais, a degradação dos nucleotídeos, a produção de aminas biogênicas, o controle bacteriológico e a avaliação sensorial.

2.1 A PESCA NO BRASIL E ESTADO DO RIO DE JANEIRO

A pescaé uma das mais antigas atividades econômicas realizadas pelo homem. Acontece tanto em escala artesanal como industrial e, quando ocorre no mar, é denominada pesca extrativa marinha e, quando em águas continentais, é denominada pesca extrativa continental (BRASIL, 2013; FAERJ/SEBRAE, 2009).

A produção brasileira de pescado, que engloba tanto a pesca extrativa quanto a aquicultura, vem apresentando aumento nos últimos anos. Em 2010, o Brasil produziu cerca de 1.264.765 toneladas, ocupando a 19º posição no cenário mundial. Em 2011, houve um aumento de aproximadamente 13% da produção, alcançando 1.432.578,0 toneladas de pescado. Deste total, 803.207,0 toneladascorrespondem ao pescado oriundo da pescaextrativa, o que coloca o Brasil na 23º colocação no “ranking” mundial (BRASIL, 2013; FAO, 2013).

A pesca extrativa marinha, em 2011, foi a principal fonte de pescado nacional, sendo responsável por 553.670 toneladas (38,7% do total de pescado), representada principalmente pela captura da sardinha-verdadeira que apresentou o maior volume de desembarque (75.122,5 toneladas), seguida da corvina (43.369,7 toneladas). A pesca extrativa continental contribui com 17,4% da produção total (249.600,2 toneladas). Nesse mesmo ano, a Região Sudeste do Brasil registrou uma produção de 114.877,3 toneladas de pescado proveniente da pesca extrativa e o Estado do Rio de Janeiro foi o principal responsável por esta produção (78.933,0 toneladas), sendo ainda o terceiro maior estado na produção nacional, contribuindo com 14,3% do total (BRASIL, 2013).

O Estado do Riode Janeiro, com 27 municípios costeiros, possui uma das mais extensas linhas costeiras do país (635 km), ocupando a terceira posição e extensão. A faixa costeira fluminense possui uma diversidade geográfica, que aliada às características oceanográficas do litoral, como a ocorrência da ressurgência, fenômeno natural de afloramento de águas frias e ricas em nutrientes que enriquece as águas da plataforma costeira, mantém a alta diversidade e produtividade de espécies pesqueiras nesta região. A região Metropolitana é a que apresenta o principal porto de desembarque do Estado. Em 2011, foram desembarcadas cerca 34 mil toneladas de pescado o que equivale a 43% da produção estadual. A sardinha verdadeira foi o principal recurso pesqueiro desembarcado na região, representando 57% da produção estadual, e o bonito-listrado ocupou a segunda posição (5%), seguido da sardinha-laje (3%), savelha (3%), galo (2%), dourado (2%), xerelete (2%), corvina (2%) e atuns (1%). A maioria destes peixes é comercializada na forma inteira e refrigerada (FIPERJ, 2011).

Em função da característica multifrota-multiespécie das pescarias da região Sudeste-Sul do Brasil, é comum algumas espécies serem capturadas por mais de um método de pesca (FAERJ/SEBRAE, 2009). No entanto, as frotas atuantes no Estado do Rio de Janeiro praticam predominantemente a pesca com barcos de cerco, principalmente porque este é o método de captura de cinco das principais espécies capturadas no Estado, entre as quais se destaca a sardinha verdadeira; o arrasto duplo que engloba a captura de espécies diversas, tais como o camarão rosa oceânico e sua fauna acompanhante, formada pela corvina, trilha, pescadinha e outras espécies demersais; barcos atuneiros, atuando com o método de captura com isca viva, para a captura do bonito listrado e da albacora laje; barcos de linha de mão e espinhel vertical, direcionados para a captura de “peixes finos”, tais como o cherne, badejo, garoupa, namorado e batata (FIPERJ, 2011; JABLONSKI; DU MONT; OLIVEIRA, 1997).

2.1.1 Tainha (*Mugil platanus*)

A tainha (*Mugil platanus*) é uma espécie pelágica pertencente à família *Mugilidae* com ampla distribuição, ocorrendo em águas tropicais e subtropicais de todo o mundo, principalmente nas regiões costeiras e estuarinas (MENEZES, 1983;

MIRANDA; CARNEIRO, 2007). No entanto, no Atlântico Sul Ocidental, essa espécie está presente apenas entre o litoral do Rio de Janeiro (23ºS) e águas argentinas e, em maior quantidade entre os meses de maio e agosto (IBAMA, 2007). Há controvérsias sobre os locais de desova da tainha no litoral brasileiro, no entanto, a maioria dos estudos indica que as tainhas adultas, com as gônadas em fases iniciais de maturação, normalmente deslocam-se pelas águas estuarinas simultaneamente à maturação das gônadas. Quando as gônadas estão maturas, a tainha aguarda as condições climáticas ideais para deixar o estuário, procurando o oceano para desovar. A desova raramente ocorre no estuário embora sejam encontradas fêmeas com as gônadas em estágios avançados de maturação, principalmente durante o período de migração reprodutiva para o mar. Os ovos fertilizados dão origem aos filhotes (juvenis) que permanecem no mar até atingirem mais ou menos 5 cm. Quando vão para o estuário, localizam-se principalmente em águas costeiras, onde a captura do alimento é mais fácil (GONZÁLES-CASTRO; MACCHI; COUSSEAU, 2011; IBAMA, 2007; MENEZES, 1983; VIEIRA; SCALABRIN, 1991).

A tainha apresenta importância comercial expressiva nas regiões Sul e Sudeste brasileiras, sendo capturada comercialmente desde o Rio Grande do Sul até o Rio de Janeiro (MIRANDA; MENDONÇA; CERGOLE, 2006, PINHEIRO et al., 2010; PORCHER et al., 2010). No Brasil, em 2011, a tainha esteve entre as cinco espécies de peixes marinhos mais capturadas, alcançando um total de 18.045,9 toneladas o que corresponde a aproximadamente 3,8% do total de peixes marinhos capturados no país (BRASIL, 2013). No Estado do Rio de Janeiro, em 2007, foram capturadas 1.903,5 toneladas de tainha, sendo esta espécie uma das que contribuíram no crescimento da produção total de pescado marinho estadual nesse ano (IBAMA, 2007), no entanto a aquisição*per capita* da tainha fresca ainda é baixa (0,033 kg/hab entre 2008 e 2009) (IBGE, 2010). Neste Estado, essa espécie pode ser encontrada no complexo lagunar de Araruama e em mar aberto na Região dos Lagos, na região entre Maricá e Itaipu e na Baía de Sepetiba. A captura pode ser realizadade diversas formas, tais como ganchos, redes de emalhe ou de espera, pequenos cercos e linhas de mão (FAERJ/SEBRAE, 2009).

Após o ano de 2000, com a diminuição da pesca da sardinha-verdadeira (*Sardinella brasiliensis*), a frota industrial de traineiras do sul e sudeste do Brasil passou a dirigir suas capturas para espécies antes consideradas acessórias, entre

elas, a tainha. Desta forma, esta espécie tornou-se um potencial recurso alternativo bastante valorizado pelo mercado consumidor (MIRANDA; CARNEIRO, 2007; MIRANDA; MENDONÇA; CERGOLE, 2006).

2.1.2 Corvina (*Micropogonias furnieri*)

A corvina (*Micropogonias furnieri*) pertencente à família *Sciaenidae* e é uma das espécies demersais mais abundantes e intensamente exploradas da plataforma continental do sul e sudeste do Brasil. Apresenta ampla distribuição, ocorrendo entre a Península de Yucatán (Golfo do México 20ºN) e o Golfo de San Matias (Argentina, 41ºS) (HAIMOVICI; IGNÁCIO, 2005; ISAAC, 1988). Normalmente é encontrada sobre fundos lodosos e arenosos em águas costeiras e em estuários onde há alimentos. Os adultos formam cardumes, alimentam-se de organismos bentônicos e ocasionalmente capturam peixes. Os juvenis alimentam-se de crustáceos e moluscos bentônicos. Os períodos de reprodução ocorrem em épocas distintas ao longo da costa, possivelmente em função das diferentes condições ambientais, tais como temperatura da água e disponibilidade de alimentos (ISAAC, 1988).

Na década de 70 a maior parte dos desembarques da corvina provinha da pesca industrial de arrasto e da pesca artesanal, principalmente com redes de emalhe. Com o desenvolvimento da frota costeira em meados da década de 1980, começou a predominar a pesca de emalhe (HAIMOVICI; IGNÁCIO, 2005). No Estado do Rio de Janeiro, a corvina é encontrada em diversas regiões, sendo capturada em sua maioria por redes de emalhe nas regiões de Cabo Frio, Baía de Sepetiba e Norte do Estado, entre a Barra de Itabapoana e Macaé, podendo ainda a pesca ocorrer com auxílio de ganchos, redes de espera e linhas-de-mão na região entre Maricá e Itaipu e por arrastos de fundo em Paraty (FAERJ/SEBRAE, 2009).

A corvina é a segunda espécie mais capturada no Brasil em 2011, com 43.369,7 toneladas (BRASIL, 2013). No Estado do Rio de Janeiro esta espécie foi a segunda mais capturada em 2007, com 10.064,5 toneladas (IBAMA, 2007) e entre 2008 e 2009, foi o peixe com maior aquisição *per capita* neste Estado (0.288 kg/hab) (IBGE, 2010).

2.2 CONTROLE DA QUALIDADE DO PEIXE FRESCO

O controle de qualidade consiste em técnicas e atividades operacionais que são utilizadas para satisfazer as exigências de qualidade (SCIORTINO; RAVIKUMAR, 1999). Para Connell (2001), de uma forma geral qualidade poderia ser definida como “grau de excelência”. Esse autor destaca ainda que, do ponto de vista do cliente, alguns dos fatores mais importantes que determinam a qualidade são facilidade de preparação, aparência, odor, sabor, frescor, tamanho, embalagem, composição, entre outros.

A avaliação da qualidade do peixe fresco pode ser realizada por testes sensoriais ou instrumentais. Os testes sensoriais são amplamente utilizados na indústria e mercado por serem rápidos e fáceis de serem executados, no entanto apresentam como desvantagem possíveis variações nos resultados em função das diferentes percepções dos avaliadores. Os métodos instrumentais, apesar de mais caros e elaborados, podem ser utilizados quando resultados precisos são necessários e ainda em complementação às avaliações sensoriais (CHENG et al., 2013; CONNELL, 2001; VARDANIS et al., 2011).

2.2.1 Fatores que afetam a qualidade do peixe

O peixe é um dos alimentos mais susceptíveis à deterioração. De forma geral, fatores intrínsecos específicos do peixe tais como sua natureza pecilotérmica, elevado pH *post mortem*, presença de grandes quantidades de nitrogênio não proteico, como o óxido de trimetilamina, elevado teor de gorduras insaturadas facilmente oxidáveis, atividade de água elevada e o baixo teor de tecido conjuntivo influenciam intensamente a microbiota facilitando a degradação deste alimento (GRAM; HUSS, 1996). No entanto, a velocidade do processo de degradação do peixe depende de fatores intrínsecos à espécie, tais como idade, tamanho, época de captura, tipo de alimentação, estado nutricional e condições fisiológicas. Além disso, o estresse na captura, as más condições de manipulação entre a captura e a entrega do produto ao consumidor, os inadequados padrões de higiene e, sobretudo, o excessivo tempo e temperatura de estocagem podem contribuir para a

perda da qualidade e aceleração do processo de deterioração (CONNELL, 2001; MACAGNANO et al., 2005; PEREIRA; TENUTA-FILHO, 2005).

O controle de temperatura, um dos itens das boas práticas de fabricação, deve ser realizado durante as etapas de produção e cadeia de distribuição, no entanto, flutuações de temperatura ocorrem, como por exemplo, devido a inadequada cobertura da matéria-prima com gelo. Neste caso, ocorrerá aceleração da taxa de degradação do peixe causada pelo crescimento microbiano e mudanças oxidativas e consequentemente diminuição da validade comercial desta matriz alimentar (ABABOUCH et al., 1996; CHONG et al., 2012; OLAFSDOTTIR et al., 2006; SONG et al., 2012; TAOUKIS; KOUTSOUMANIS; NYCHAS, 1999; VECIANA-NÓGUES; MARINE-FONT; VIDAL- CAROUL, 1997).

2.2.2 Parâmetros de qualidade oficiais

Alguns parâmetros bacteriológicos, sensoriais e físico-químicos de qualidade são estabelecidos por órgãos reguladores em todos os países. Normas nacionais para padrões bacteriológicos, para avaliação da qualidade do pescado resfriado são descritos na Resolução da Diretoria Colegiada – RDC nº 12 (BRASIL, 2001) que estabelece limite máximo apenas para a contagem de estafilicocos coagulase positivo e caso haja presença de *Salmonella* spp. a amostra é caracterizada como imprópria para o consumo. A legislação nacional não prevê limite para a contagem de bactérias heterotróficas aeróbias mesófilas e psicrotróficas, que é de suma importância por avaliarem a qualidade sanitária e o grau de deterioração desta matriz alimentar (JAY, 2005). No entanto, é interessante ressaltar, que cada espécie pesqueira possui microbiota específica de deterioração, sendo o número dessas bactérias, e não o número total de microrganismos, que estabelecerá relação com a duração do tempo de estocagem do produto (HUSS, 1999). Daí a necessidade de correlação dos resultados com outros testes laboratoriais.

Para a avaliação sensorial, o Decreto nº 30.691 (BRASIL, 1952), a Portaria nº 185 (BRASIL, 1997) e a Instrução Normativa nº 25 (BRASIL, 2011) estabelecem que o peixe fresco deva apresentar-se íntegro; com odor, cor e sabor próprios; com escamas translúcidas e brilho metálico, unidas entre si e fortemente aderidas à pele, não devendo estar viscosas; pele úmida, tensa e bem aderida; mucosidade,em

espécies que a possuem, aquosa e transparente; olhos vivos, brilhantes e salientes, devendo ocupar a cavidade orbitária; ânus fechado; curvatura natural do corpo; opérculo rígido com resistência à abertura; brânquias de coloração rósea a vermelho intenso, úmidas e brilhantes, ausência ou discreta presença de muco; abdômen tenso, sem diferença externa com a linha ventral; carne firme, de consistência elástica; músculos aderidos fortemente aos ossos da espinha dorsal e/ou cartilagens, de elasticidade marcante; vísceras íntegras, perfeitamente diferenciadas, brilhantes e sem dano aparente; e face interna nacarada, os vasos sanguíneos cheios e fixos. Com o processo de deterioração, o pescado vai perdendo essas propriedades sensoriais características, tornando-se um produto de baixa qualidade. No entanto, um fator relevante é que há alterações que ocorrem no processo de deterioração do peixe que são espécie-específicas o que torna a avaliação sensorial baseada na legislação frágil do ponto de vista aplicativo.

Para os parâmetros físico-químicos o Decreto nº 30.691 (BRASIL, 1952), a Portaria nº 185 (BRASIL, 1997) estabelecem limites apenas para bases voláteis totais e terciárias, pH da carne externa e interna, reação de gás sulfídrico e teor de histamina para as espécies pertencentes às famílias *Scombriade*, *Scombrisocidae*, *Clupeidae*, *Coryyhaenidae* e *Pomatomidae*. No entanto, não considera metabólitos importantes formados na etapa inicial do processo de deterioração do pescado como os produtos da degradação do ATP e a presença de outras aminas biogênicas, além da histamina (GRAM; DALGAARD, 2002; ÖZOGUL; ÖZOGUL; KULEY, 2008). A Instrução Normativa nº 25 (BRASIL, 2011) fixa metodologias analíticas para avaliação da qualidade do pescado e inclui a determinação de aminas biogênicas histamina, putrescina, cadaverina, espermidina e espermina por cromatografia líquida de alta eficiência, entretanto os limites de aceitabilidade ainda não foram estabelecidos, com exceção da histamina, cujo limite já havia sido anteriormente estabelecido pela Portaria nº 185 (ibid).

2.3 ÍNDICES BIOQUÍMICOS DE QUALIDADE

2.3.1 Degradação de nucleotídeos

A degradação dos nucleotídeos pode refletir as primeiras alterações no pescado antes do crescimento bacteriano. No entanto, a taxa e o padrão de degradação podem variar em função da espécie, tipo de músculo, condição biológica dos exemplares (sexo, estado gonodal), estação do ano, temperatura da água, método de captura e condições de estresse durante a captura, manuseio e armazenamento (HUSS, 1999; LUONG et al., 1992; MORKORE et al., 2010; ÖZYURT et al., 2007; VALLS; DELGADO, 2000; VECIANA-NOGUÉS; MARINE-FONT; VIDAL CAROUL, 1997).

Logo após a captura e morte do pescado, por ação de enzimas presentes no músculo, aadenosina trifosfato (ATP) é convertida, por reações de desfosforilação, em adenosina difosfato (ADP) e, posteriormente, em adenosina monofosfato (AMP). O AMP por sua vez é desaminado a inosina monofosfato (IMP) que se degrada a inosina (HxR) e hipoxantina (Hx) (CONTRERAS-GUZMÁN, 1994; HUSS, 1999; OCAÑO-HIGUERA et al., 2009; SONG et al., 2012; VALLS; DELGADO, 2000). Para invertebrados marinhos, há um mecanismo alternativo que considera uma sequência de desfosforilação até adenosina (Ade), que posteriormente se degrada a HxR e Hx e, em alguns casos, pode formar xantina e ácido úrico (SAITO; AARAI; MATSUYOSHI, 1959; HUSS, 1999; VENUGOPAL, 2002). Desta forma, a degradação dos nucleotídeos em pescado pode seguir duas vias, uma que envolve a formação de inosina monofosfato e outra que considera uma sequência de desfosforilação até adenosina, no entanto em algumas espécies podem ocorrer as duas vias eem outras, pode haver a prevalência da formação de adenosina, ocorrendo acúmulo de AMP e ausência de IMP (CONTRERAS-GUZMÁN, 1994).

Em função da variabilidade de metabolização de HxR a Hx, Ehira; Uchiyama (1973, 1987) propuseram uma classificação de acordo com o valor da relação HxR:Hx, agrupando as distintas espécies pesqueiras que apresentam HxR:Hx ≥ 5:1 no grupo A; as que apresentam HxR:Hx entre 5:1 e 1:5 no grupo B; e no grupo C as espécies que apresentam HxR:Hx ≤ 1:5.

A conversão de ATP a IMP ocorre rapidamente pela ação de enzimas endógenas presentes no músculo. No entanto a degradação do IMP a HxR e Hx é mais lenta e ocorre por ação de enzimas autolíticas e microbianas, sendo a taxa de formação por bactérias superior a autolítica. Desta forma o IMP é acumulado na etapa inicial de degradação, sendo o principal responsável pela definição do aroma e sabor do peixe fresco. À medida que a qualidade do peixe decresce verifica-se aumento dos níveis de HxR, que é mais ou menos insípida, e Hx que tem efeito direto sobre o sabor amargo dos peixes em deterioração (CONTRERAS-GUZMÁN, 1994; GRAM; HUSS, 1996; MASSA; PAREDI; CRUPKIN, 2002; MASSA et al., 2005). Em algumas espécies a HxR pode desaparecer rapidamente causando o acúmulo de Hx, no entanto em outras a HxR é modificada lentamente, permanecendo sem alteração por vários dias (CONTRERAS-GUZMÁN, 1994; ÖZOGUL; ÖZOGUL; KULEY, 2008; SONG et al., 2012).

Desta forma, os produtos originados da degradação dos nucleotídeos têm sido amplamente utilizados como indicadores da qualidade (AUBOURG et al., 2007; LI; LI; HU, 2013;ÖZOGUL et al., 2006; ÖZOGUL; ÖZOGUL; KULEY, 2008; RZEPKA et al., 2013; SONG et al., 2012).

2.3.1.1 Índices de qualidade

Baseado na concentração dos diferentes nucleotídeos, o valor K foi proposto por Saito, Aarai e Matsuyoshi (1959) como índice de qualidade para avaliar o grau de frescor do pescado sendo definido como:

$$K\ (\%) = \frac{HxR + Hx}{ATP + ADP + IMP + HxR + Hx}\ x\ 100$$

Esses autores propuseram uma classificação para as espécies comerciais de pescado de acordo com os valores K obtidos. Para valores K < 20% o pescado seria considerado muito fresco, inclusive apto para consumo cru teria; para valores K entre 20 e 40% o pescado seria classificado apenas como fresco podendo ser consumido após o cozimento; e o pescado com valores K > 40% seria considerado impróprio para o consumo. Gonçalves (2011), a partir da aplicação desta fórmula,

propôs uma nova classificação, considerando como fresco (recém-abatido com morte sem sofrimento) o pescado com valores K < 5%; fresco podendo ser utilizado ainda para preparo de sushi e sashimi o pescado com valores 5% ≤ K ≤ 20%; valores 20% ≤ K ≤ 60% para pescado que deve ser submetido a cocção antes de ser consumido/processado; e valores 60% ≤ K ≤ 80% para pescado com sinais de putrefação.

Como a passagem de ATP a IMP é rápida, Karube et al. (1984) ao avaliarem diferentes espécies de peixe estocadas em gelo por duas semanas, propuseram um novo cálculo (Ki) que não considera os compostos ATP, ADP e AMP sendo este mais adequado para avaliação do grau de frescor das diferentes espécies de peixe capturadas e comercializadas no Estado do Rio de Janeiro, visto que devido às condições de captura associada ao clima da região, a degradação desses compostos ocorre rapidamente não sendo possível a quantificação dos mesmos. Sendo assim, o valor Ki seria definido como:

$$Ki\ (\%) = \frac{HxR + Hx}{IMP + HxR + Hx}\ x\ 100$$

Burns, Ke e Irvine (1985), ao trabalharem com amostras de bacalhau, cavala e caranguejo, propuseram o valor G baseado no acúmulo de Hx, mas reflete a degradação de IMP, AMP e HxR, de acordo com a seguinte fórmula:

$$G(\%) = \frac{Hx + HxR}{HxR + IMP + AMP}\ x\ 100$$

O valor H foi proposto por Luong et al. (1992), para espécies de pescado nas quais ocorre um rápido acúmulo de HxR e Hx. Esses autores relataram que, nestes casos, os valores K e Ki aumentam de forma rápida e logo se mantém mais ou menos constante, não refletindo adequadamente as alterações que ocorrem. O valor H baseia-se na concentração de Hx, considerado um bom indicador do frescor do pescado, tanto sob o ponto de vista fisiológico, como pelo sensorial, em função do sabor amargo característico do pescado em deterioração:

$$H(\%) = \frac{Hx}{IMP + Hx + HxR}\ x\ 100$$

Diversos estudos que demonstram a aplicabilidade desses índices têm sido conduzidos ao longo dos anos. Lakshmanan, Antony e Gopakumar (1996), ao calcularem o valor K para duas espécies de peixe (*Liza corsula* e *Etroplus suratensis*) estocadas em temperatura ambiente e em gelo, relataram divergências com os limites propostos por Saito, Aarai e Matsuyoshi (1959), pois os peixes mantidos em temperatura ambiente foram rejeitados apenas com valores K superiores a 60%. As amostras de *Liza corsula* e *Etroplus suratensis* estocadas em gelo foram classificadas como de primeira qualidade para valores K de 29,8% e 23,5%, boas com valor K de 50%, sendo rejeitadas com valor K de 70,59% e 54,94%, respectivamente. Özogul; Özogul; Kuley (2008), Özogul et al. (2011) e Song et al. (2012) sugeriram um valor K de 81%,80% e 60% como limite de aceitabilidade para amostras de garoupas (*Epinephelus aeneus*), linguado (*Solea sole*) e carpas (*Megalobrama amblycephala*), valores estes superior ao sugerido por Saito, Aarai e Matsuyoshi (1959).

Huidobro, Pastor e Tejada (2001) ao avaliarem douradas (*Sparus aurata*) evisceradas e inteiras obtidas por diferentes métodos de sacrifício (imersão em água e gelo, asfixia, anestesia com posterior percussão e percussão) observaram que o valor K entre 50-60% foi alcançado quando as avaliações sensorial e microbiológica demonstraram valores já compatíveis com perda de qualidade, corroborando com o proposto por Saito, Aarai e Matsuyoshi (1959). Em espécimes de matrinxã (*Brycon cephalus*) mantidos em gelo, Batista et al. (2004) observaram que nos primeiros dias de armazenamento não ocorreu variação expressiva para o índice K (2,01 a 3,29%) e que, após 16 dias, o valor K alcançou 19,56%, mantendo correlação significativa com os parâmetros sensoriais, físico-químicos e bacteriológicos, sendo o peixe considerado adequado ao consumo. Li, Li e Hu (2013) observaram que o valor K apresentou boa correlação com a perda de qualidade da corvina amarela (*Pseudosciaena crocea*) estocada a 4ºC e queos limites propostos por Saito, Aarai e Matsuyoshi (1959) foram adequados, pois no dia 0 de estocagem o valor K foi aproximadamente 10% alcançando 55% no 20º dia de estocagem.

Özogul et al. (2006) e Song et al. (2012) calcularam os valores K, Ki, H e G para robalos (*Dicentrarchus labrax*) estocados a 4ºC e em gelo e carpas (*Megalobrama amblycephala*) estocadas refrigeradas e parcialmente congeladas, respectivamente, e observaram aumento contínuo desses índices ao longo do período de armazenamento. Corroborando com os achados de Özogul; Özogul (2002), verificaram que o valor H subiu lentamente devido ao aumento constante da concentração de Hx. Além disso, os autores relataram que o rápido aumento dos valores K, Ki e G ocorreu devido à queda acentuada de IMP, não sendo observada diferença significativa entre esses valores. Resultados semelhantes foram observados por Özogul, Özogul e Gökbulut (2006) na avaliação de enguia (*Anguilla anguilla*) e Özogul; Özogul; Kuley (2008) na análise de garoupas (*Epinephelus aeneus*).

2.3.1.2 Métodos de análise

Para determinação dos nucleotídeos, muitos métodos têm sido relatados, tais como a utilização de colunas de troca cátion-iônica (SAITO; AARAI; MATSUYOSHI, 1959), sistema sensor de enzimas (KARUBE et al., 1984), eletroforese capilar (LUONG et al., 1992) e a Cromatografia Líquida de Alta Eficiência (CLAE) sendo esta técnica a mais frequentemente descrita nos diversos estudos (BURNS; KE; IRVINE, 1985; HUIDOBRO; PASTOR; TEJADA, 2001; LAKSHMANAN; ANTONY; GOPAKUMAR, 1996; ÖZOGUL et al., 2000; ÖZOGUL et al., 2006; ÖZOGUL et al., 2011; ÖZOGUL; ÖZOGUL; KULEY, 2008; SIRIPATRAWAN; SANGUANDEEKUL; NARAKAEW, 2009; URIARTE-MONTOYA et al., 2010; VALLS-DELGADO, 2000; VÁZQUEZ-ORTIZ et al., 1997). Para a fase de extração dos compostos, o reagente ácido perclórico é o mais utilizado. A separação dos compostos é comumente realizada em colunas de fase reversa usando uma fase móvel de tampões fosfatos ou por métodos íon-pariônicos. Solventes orgânicos tais como metanol e acetonitrila podem ser utilizados para diminuir o tempo de corrida cromatográfica, conforme sugerido por Özogul et al.(2000).

2.3.2 Produção de aminas biogênicas

Aminas biogênicas são compostos orgânicos de caráter básico e baixo peso molecular formadas por reações de descarboxilação de aminoácidos precursores ou por aminação e desaminação de aldeídos e cetonas e sintetizadas no metabolismo de plantas, animais e microrganismos (BARDÓCZ, 1995; BRINK et al., 1990;SILLAS SANTOS, 1996).

De acordo com a estrutura química, as aminas podem ser classificadas em alifáticas (putrescina, cadaverina e agmatina), aromáticas (tiramina, β-feniletilamina) e heterocíclicas (histamina, triptamina). Conforme o número de grupamentos aminas, são ainda divididas em monoaminas (tiramina, β-feniletilamina), diaminas (histamina, triptamina, serotonina, putrescina e cadaverina) e poliaminas (agmatina). Quanto à via de formação, em naturais e biogênicas. As naturais são formadas *in situ* nas células a partir de uma molécula mais simples à medida que são requeridas, como no caso espermina e espermidina que são formadas a partir da putrescina pela adição de um ou dois grupos aminopropil, respectivamente. As biogênicas são formadas pela ação das enzimas descarboxilases dos microrganismos a partir dos aminoácidos precursores. Neste grupo se incluem a tiramina, histamina, triptamina, β-feniletilamina, putrescina, cadaverina e agmatina. A histamina também pode ser classificada como natural, visto que está armazenada nos mastócitos e basófilos ou biogênicas (BARDÓCZ, 1995; SHALABY, 1996; SILLAS SANTOS, 1996).

Embora consideradas endógenas em certos alimentos, estando presentes em baixas concentrações, as aminas biogênicas normalmente são formadas como resultado da ação bacteriana associada à disponibilidade de aminoácidos livres, condições favoráveis ao crescimento bacteriano e produção de enzimas (CARDOZO et al., 2013; SILLAS SANTOS, 1996; SILVA et al., 2011). A quantidade e o tipo de amina biogênica formada dependem da composição do alimento e do tipo de microrganismo presente (BRINK et al., 1990).

Numerosos gêneros bacterianos presentes nos alimentos têm capacidade aminoácido descarboxilase, tais como *Bacillus*, *Citrobacter*, *Clostridium*, *Klebsiella*, *Escherichia*, *Proteus*, *Pseudomonas*, *Salmonella*, *Shigella*, *Photobacterium*, *Lactobacillus*, *Pediococcus* e *Streptococcus*. Esses microrganismos podem estar naturalmente presentes nos alimentos, serem adicionados intencionalmente ou

serem introduzidos por contaminação, como ocorre nos casos de manipulação inadequada antes, durante e após o processamento do alimento (BRINK et al., 1990; EHSANI; JASOUR, 2012). A espécie *Morganella morganii*, determinadas cepas de *Klebsiella pneumoniae* e algumas de *Hafnia alvei* são prolíferas produtoras de histamina, apresentando especial importância em pescado (SILLAS-SANTOS, 1996).

Dentre os produtos de origem animal, os peixes são os mais susceptíveis à formação de aminas biogênicas, principalmente de histamina. Isso se deve a algumas espécies, especialmente as pertencentes à família *Scombridae*, tais como atum e bonito, e àqueles da família *Clupeidae* como as sardinhas, conterem elevada quantidade de histidina livre na musculatura (OLIVEIRA et al., 2004; RZEPKA et al., 2013). O teor de aminas biogênicas presente no peixe pode variar de acordo com a época do ano, genética, ambiente, alimentação, sexo, estágio fisiológico, período de armazenamento e tecido amostrado (ABABOUCH et al., 1996; LEE et al., 2012;VECIANA-NÓGUES; MARINE-FONT; VIDAL-CAROUL, 1997), sendo a formação influenciada diretamente pela temperatura de estocagem (CARMO et al., 2010;SILVEIRA et al., 2001).

As aminas biogênicas geralmente não representam risco à saúde humana, pois são rapidamente metabolizadas através de reações de acetilação e oxidação mediadas por enzimas monoamino-oxidase, diamino-oxidase, poliamino-oxidase e histamina N-metiltransferase (BARDÓCZ, 1995; HUSS, 1997; LEHANE; OLLEY, 2000). Portanto, as intoxicações por aminas biogênicas ocorrem quando são ingeridas em grande quantidade, quando o mecanismo natural de catabolismo do indivíduo é geneticamente deficiente ou inibido por agentes farmacológicos ou, quando substâncias potencializadoras estão presentes (LEHANE; OLLEY, 2000; ÖNAL, 2007; SHALABY, 1996).

A intoxicação alimentar mais frequentemente observada é causada pela reação alérgica a histamina, que se caracteriza por um curto período de incubação e duração, e aparecimento dos seguintes sintomas: diminuição da pressão sanguínea, urticária, cefaléia, palpitações cardíacas; tonturas; desfalecimento; secura na boca e garganta; eritema no rosto e pescoço, disfagia, podendo ocorrer choque anafilático (SHALABY, 1996). Cadaverina e putrescina são exemplos de aminas biogênicas potencializadoras da ação da histamina, pois inibem o sistema de detoxificação do

indivíduo e aumentam a absorção intestinal da histamina. Além disso, podem ainda agir como precursores de nitrosaminas formando compostos carcinogênicos (OLIVEIRA et al., 1995). Outra amina de suma importância é a tiramina, responsável pela crise hipertensiva causada, na maioria das vezes, pelo consumo concomitante de alimentos contendo esta amina e o uso de medicamentos inibidores da monoamino-oxidase, sendo também desencadeadora de crises de enxaqueca (BRINK et al., 1990; ÖNAL, 2007).

As aminas biogênicas, além de apresentarem importância do ponto de vista toxicológico, podem ser usadas para estimar o frescor ou o grau de deterioração do pescado, pois são compostos presentes em baixos níveis no pescado fresco cuja produção está associada à atividade de bactérias deterioradoras (LAPA-GUIMARÃES, 2004; VECIANA-NÓGUES; MARINE-FONT; VIDAL-CAROUL, 1997). Em alimentos processados termicamente, a presença destes compostos indica alteração da matéria prima e pode, inclusive, apresentar incremento nos níveis durante período de estocagem, visto que a maioria das aminas é estável ao calor e algumas descarboxilases permanecem ativas mesmo após a o processamento térmico (BRINK et al., 1990; HUSS, 1999).

2.3.2.1 Índices de aminas biogênicas

Um dos primeiros índices de qualidade baseado na concentração de aminas biogênicas foi proposto por Mietz e Karmas (1977). Esses autores analisaram o frescor do atum que seria utilizado na elaboração de conservas e observaram que os níveis de putrescina, cadaverina e histamina aumentavam, enquanto os de espermina e espermidina diminuíam nas amostras deterioradas em comparação com as amostras de boa qualidade. Desta forma propuseram um índice de qualidade química baseado na concentração dessas aminas biogênicas:

$$IQ = \frac{histamina + putrescina + cadaverina}{espermina + espermidina}$$

Os autores observaram que aumento do índice de qualidade ocorria à medida que a pontuação sensorial do produto enlatado diminuía. Sendo assim, sugeriram

que um produto com IQ inferior a 1 seria considerado de primeira qualidade, enquanto que aqueles com valores acima de 10 indicariam uma qualidade microbiológica muito ruim.Para outras espécies de pescado, esse índice apresentou adequada correlação com o grau de frescor, conforme observado por Özogul; Özogul (2006) e Bakar et al. (2010) em amostras de sardinhas (*Sardina pilchardus*) mantidas a 4ºC em ar, embaladas em atmosfera modificada (60% CO_2 e 40% N_2) e a vácuo e perca-gigante (*Lates calcarifer*) estocada a 0ºC e 4ºC, respectivamente. Entretanto, resultados divergentes foram observados em outros estudos. Em atuns estocados a 8ºC, Veciana-Nógues, Marine-Font e Vidal Caroul (1997) observaram que a rejeição sensorial ocorreu antes do valor IQ igual a 10 ter sido alcançado. Em carpas (*Cyprinus carpio*) estocadas em embalagens de polietileno não hermética mantidas a 3 e 15ºC, Křížek, Pavlíček e Vácha (2002) observaram que a concentração de espermina não declinou no decorrer do período de estocagem e, por este motivo, o índice IQ não apresentou aplicabilidade adequada para avaliação desta espécie.

Outros índices de qualidade baseados na concentração de aminas biogênicas, individual ou conjuntamente, têm sido propostos ao longo dos anos. Veciana-Nógues, Marine-Font e Vidal-Caroul (1997) propuseram que a soma dos níveis de histamina, cadaverina, putrescina e tiramina seria indicadora da qualidade de atuns. Esses autores observaram que em atuns estocados em diferentes temperaturas (0, 8 e 20ºC), os níveis de tiramina, cadaverina, putrescina e histamina aumentaram. Desta forma, consideraram que os valores obtidos abaixo de 50 $mg.kg^{-1}$, indicariam um alimento de boa qualidade. Özogul; Özogul (2006) e Bakar et al. (2010) observaram que esse índice também apresentou boa correlação com o grau de frescor de sardinhas (*Sardina pilchardus*) mantidas a 4ºC em ar, embaladas em atmosfera modificada (60% CO_2 e 40% N_2) e a vácuo e perca-gigante (*Lates calcarifer*) estocada a 0ºC e 4ºC, respectivamente. Em lulas e salmão, Yamanaka, Shiomei e Kikuchi (1989) propuseram como indicadores de qualidade os níveis de agmatina e cadaverina, respectivamente. Ruiz-Capilas e Moral (2001) relataram que os teores de cadaverina e agmatina são bons indicadores da qualidade de pescada-branca. Křížek, Pavlíček e Vácha (2002), Rezaei et al. (2007) e Rodrigues et al., (2013) propuseram que a soma dos teores de putrescina e cadaverina poderia ser

promissora para avaliação da qualidade da carpa (*Cyprinus carpio*) e trutas (*Oncorhynchus mykiss*).

2.3.2.2 Métodos de análise

Vários métodos analíticos têm sido desenvolvidos para a avaliação de aminas biogênicas em pescado. Espectrofluorimetria, cromatografia em camada delgada, cromatografia líquida de alta eficiência (CLAE), cromatografia gasosa (CG), eletroforese capilar e a reação em cadeia polimerase (PCR) são citados como os mais relevantes sob o ponto de vista analítico (LAPA-GUIMARÃES; PICKOVA, 2004; ÖNAL, 2007; SHALABY, 1996; 1999).

A espectrofluorimetria é considerada como método oficial pela AOAC nos EUA e adotado como tal no Brasil para a análise quantitativa de histamina em peixes (AOAC, 2002; BRASIL, 1997). Entretanto, a Cromatografia Líquida de Alta Eficiência (CLAE), método oficial estabelecido pela União Europeia (COMUNIDADE EUROPEIA, 2005) para determinação de histamina em pescado e derivados, tornou-se também o método oficial no Brasil para determinação de histamina e outras aminas biogênicas (cadaverina, putrescina, espermidina, espermina) em pescado através da Instrução Normativa nº 25 (BRASIL, 2011). A técnica consiste na extração ácida das aminas, derivatização antes da coluna e fora de linha com cloreto de dansila em pH alcalino, seguido de separação e quantificação por CLAE com gradiente de eluição e detecção ultravioleta. De acordo com Shalaby (1996) a CLAE é uma técnica seletiva e sensível que, segundo Park et al. (2010) é a mais utilizada para determinação de aminas biogênicas devido a alta resolutividade.

A cromatografia em camada delgada é um método semi-quantitativo simples, rápida e de baixo custo para separar e estimar aminas biogênicas nos alimentos (ANDRADE et al., 2010; ANDRADE et al., 2012; CARMO et al., 2010;LAPA-GUIMARÃES; PICKOVA, 2004; MONTEIRO; MÁRSICO; VITAL, 2010; ÖNAL, 2007; SHALABY, 1996; 1999) utilizado na rotina do controle de qualidade de indústrias e entrepostos.

Para a fase de extração dos compostos, os solventes comumente utilizados são ácido tricloracético (ANDERSON, 2008; BAKAR et al., 2010; MENDES et al., 1999; OLIVEIRA et al., 2004; ÖZOGUL; ÖZOGUL; KULEY, 2008; ÖZOGUL;

ÖZOGUL, 2006; ÖZOGUL; ÖZOGUL; GÖKBULUT, 2006; ÖZOGUL et al., 2006; PACHECO-AGUILAR et al., 1998; REZAEI et al., 2007; ÖZOGUL; RUIZ-CAPILLAS; MORAL, 2001; SHALABY, 1999), ácido perclórico (DADÁKOVÁ; KŘIŽEK; PELIKÁNOVÁ, 2009; KIM; MAH; HWANG, 2009; KŘÍŽEK; PAVLÍČEK; VÁCHA, 2002; SHAKILA; VIJAYALAKSHMI; JEYSASEKARAN, 2003; YAMANAKA; SHIOMEI; KIKUCHI, 1989) e metanol (ANDRADE et al., 2010; ANDRADE et al., 2012; DU et al., 2002; KIM et al., 2001).

Devido à falta de cromóforos e propriedades de fluorescência significativas da maioria das aminas biogênicas, a derivatização química é realizada para aumentar a sensibilidade na determinação destes compostos (ALBERTO; ARENA; NADRA, 2002; PARK et al., 2010). Normalmente as reações de derivatização ocorrem entre o grupamento amino e o agente derivatizante, sendo comumente utilizado o cloreto de dansilo, cloreto de benzoílo, fluoresceína, 9-fluorenilmetil cloroformato, naftaleno-2,3-dicarboxaldeído, o-ftalaldeído (OPA) e 6-aminoquinolil-N-hidroxisuccinimidil carbamato (AQC). A utilização do AQC foi desenvolvida por Cohen e Michaud (1993) para análise cromatográfica de aminoácidos e tem sido adaptada para análise de aminas biogênicas em diversas matrizes alimentares, constituindo um grande avanço na detecção destes compostos por, entre outros fatores, apresentar estabilidade e grande sensibilidade à detecção por fluorescência (MARTÍNEZ et al., 2000; ORDÓÑEZ et al., 2013).

2.4 PARÂMETROS BACTERIOLÓGICOS

A perda da qualidade inicial do pescado é primariamente causada por mudanças autolíticas e não relacionadas com a atividade microbiana. Essas mudanças autolíticas contribuem para a deterioração, principalmente pela formação de catabólitos disponíveis para o crescimento bacteriano (GRAM; HUSS, 1996), porém grande parte das alterações do pescado é consequência do crescimento e metabolismo dos microrganismos resultando na formação de aminas, sulfetos, alcoóis, aldeídos, cetonas e ácidos orgânicos com sabores e odores desagradáveis e inaceitáveis (ABABOUCH et al., 1996; GRAM; DALGAARD, 2002; OCAÑO-HIGUERA et al., 2011; RZEPKA et al., 2013).

Nos peixes a microbiota é encontrada geralmente na pele, nas brânquias e intestinos (JAY, 2005), sendo os músculos e líquidos corporais naturalmente estéreis (OGAWA; MAIA, 1999). No pescado de águas temperadas há predominância de bactérias Gram-negativas, da qual fazem parte as pertencentes aos gêneros *Pseudomonas*, *Moraxella*, *Shewanella*, *Flavobacterium*, *Vibrio, Acinetobacter* e *Aeromonas*, porém microrganismos Gram-positivos, tais como *Bacillus*, *Micrococcus*, *Clostridium*, *Lactobacillus* e *Corynebacterium* também podem ser encontrados em proporções variáveis. Peixes de águas tropicais podem apresentar um ligeiro aumento da carga de bactérias Gram-positivas e entéricas, porém o restante se assemelha à microbiota de peixes de águas temperadas (GRAM; HUSS, 1996; JAY, 2005).

A microbiota responsável pela deterioração do peixe fresco varia com as mudanças na temperatura de armazenamento. Em peixes refrigerados (0-5ºC), *Shewanella putrefaciens*, *Photobacterium phosphoreum*, *Aeromonas* spp. e *Pseudomonas* spp. são as principais bactérias relacionadas com a deterioração. Entretanto em temperaturas de armazenamento altas (15-30ºC) os principais responsáveis pela deterioração são as diferentes espécies da família *Vibrionanaceae*, *Enterobacteriaceae*, bem como bactérias Gram positivas (HUSS, 1999).

Os gêneros *Pseudomonas* e *Shewanella* são os mais importantes na deterioração do pescado, com destaque para as espécies *P. fluorescens*, *P. fragi* e *S. putrefaciens*, responsáveis pelas alterações sensoriais desses produtos devido à formação de trimetilamina, ésteres, substâncias voláteis redutoras e outros compostos com aroma pronunciado. Esses gêneros são importantes, devido não só à sua natureza psicrotrófica, mas, principalmente, pela capacidade que possuem em utilizar, para seu desenvolvimento, substâncias nitrogenadas não-proteicas (FRANCO; LANDGRAF, 2005).

2.5 AVALIAÇÃO SENSORIAL

Apesar das alterações químicas e microbiológicas serem de suma importância na avaliação do grau de frescor do pescado, para o consumidor, as

mudanças sensoriais como aparência, odor, textura e sabor são as primariamente percebidas e utilizadas no momento da compra.

Sendo assim, baseado na avaliação dos diversos atributos de qualidade como: aparência, textura, olhos, brânquias e abdome, e na modificação desses atributos de acordo com o tempo de estocagem, um recente esquema de avaliação sensorial do grau de frescor do pescado, o método do índice de qualidade (MIQ) está sendo frequentemente usado. Neste método, a cada atributo avaliado é dado uma pontuação, que varia de zero a três ou de zero a dois (de acordo comgrau de importância), sendo considerada zero como a melhor e três como a pior pontuação. O peixe no momento da captura tem pontuação zero ou próxima de zero. Conforme vai se deteriorando, os atributos vão adquirindo pontuações mais elevadas acumulando pontos de demérito, cujo valor máximo varia de acordo com o protocolo desenvolvido para a espécie estudada (NUNES; BATISTA, 2004; SVEINSDOTTIR et al., 2003).

O MIQ utiliza um sistema prático de classificação no qual as pontuações registradas em cada característica se somam para dar uma pontuação sensorial total, o denominado índice da qualidade (HUSS, 1999). Não é dada ênfase a um único atributo, não sendo a amostra rejeitada com base somente nos resultados obtidos em um atributo isoladamente (NUNES; BATISTA, 2004).

Este método tem sido utilizado para avaliação de várias espécies de pescado, incluindo sardinhas (*Sardina pilchardus*, *Sardinops sagax*, *Sardinella brasiliensis* e *Cetengraulis edentulus*) (ANDRADE et al., 2012; MUSGROVE et al., 2007; TRIQUI; BOUCHRITI, 2003), merluza (*Merluccius merluccius*) (BAIXAS-NOGUEIRAS et al., 2003), salmão (*Salmo salar*) (SVEINSDOTTIR et al., 2003), polvo (*Octopus vulgaris*) (BARBOSA; VAZ-PIRES, 2004), anchovas (*Engraulis encrasicholus*), (PONS-SÁNCHEZ-CASCADO et al., 2006), bacalhau (*Gadus morhua*) (BONILLA; SVEINSDOTTIR; MARTINSDOTTIR, 2007), lula (*Sepia officinalis*) (SYKES et al., 2009), camarão (*Litopenaeus vannamei*) (OLIVEIRA et al., 2009), corvina (*Micropogonias furnieri*) (TEIXEIRA et al., 2009), dourado (*Sparus aurata*) (CAMPUS et al., 2011;HUIDOBRO; PASTOR; TEJADA, 2001), filés de tilápia (*Oreochromis niloticus)* (SOARES; GONÇALVES, 2012) e pacu (*Piaractus mesopotamicus*) (BORGES et al., 2013). Entretanto, estudos que envolvem a influência da temperatura de estocagem na sua elaboração são escassos e podem fornecer

informações relevantes, na medida em que simulam situações reais e rotineiras na manipulação do pescado.

3 DESENVOLVIMENTO

3.1 ARTIGO 1: CHEMICAL QUALITY INDEXES OF MULLET (*Mugil platanus*) STORED ON ICE. Accepted for publishing by Food and Nutrition Sciences.

Chemical quality indexes of mullet (*Mugil platanus*)stored on ice

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Abstract

The present study was conducted aiming at establishing chemical quality parameters to assess ice stored mullet (0±1ºC) through the evaluation of nucleotide (adenosine monophosphate [AMP], inosine [HxR] and hypoxanthine [Hx]) degradation, biogenic amine (histamine [HI], putrescine [PU], cadaverine [CA] and tyramine [TI]) quantification and mesophilic and psychrotrophic bacteria count monitoring. The microbial load of 7 log CFU.g^{-1} established as maximum acceptable limit was attained after the 20th day of ice storage. IMP concentration declined during the storage period to levels below the detection limit. HxR content increased only up to time T3 and then declined. Hx level increased during all the storage period. CA and HI content increase was not observed, on the other hand, PU and TI contents significantly increased ($p<0.05$) at time T5. We concluded that IMP and Hx concentrations can be adequate parameters to assess mullet quality under the study conditions. HxR proved to be adequate to evaluate the freshness of mullet in the first days of storage while the amines, PU and TI, can be used to assess loss of quality. Mullet obtained in conditions similar to those of the present study and maintained at 0±1ºC can be consumed up to the 20th storage day.

Keywords

Quality parameters, biogenic amines, nucleotides, hypoxanthine, Mugilidae

1. Introduction

Fish per capita consumption has increased worldwide during the past five decades [1] due to its nutritional quality with desirable content of proteins of high biological value, quick digestibility and content of essential amino acids and poly-unsaturated fatty acids, especially the omega-3 [1,2,3]. However, due to its chemical composition, high water activity, presence of easily oxidized unsaturated fats and pH close to neutrality, this food is highly susceptible to spoilage [2,4,5] which justifies the development of species-specific researches to assess the quality of each food matrix.

Fish spoilage process begins with the action of endogenous enzymes from the muscle tissue or enzymatic activity of the viscera. The catabolites formed during this autolytic process, favor bacterial growth [5], with consequent formation of compounds with undesirable flavors and odors [6,7], frequently used to assess fish quality.

The products originated from nucleotide degradation have been used as quality indicators [8,9,10,11,12,13], because they allow detecting the initial alterations that occur immediately after fish capture. Adenosine triphosphate (ATP) is converted, by dephosphorilation in adenosine diphosphate (ADP) and adenosine monophosphate (AMP), deaminated to inosine monophosphate (IMP) which gives inosine (HxR) and hypoxanthine (Hx) as degradation products [2,2,11]. Most of these reactions occur by the action of endogenous enzymes present in the muscle, however, the degradation of IMP to HxR and Hx occurs by the action of both, autolytic and microbial enzymes [2,5]. Similarly, the characterization and the concentration of biogenic amines have been used as quality parameters [14,15,16,17], because these products are present in low levels and its formation is associated with the activity of deterioration bacteria [14,18] through decarboxylation of precursor amino acids [18,19].

In this context, due to the need of establishing reliable parameters for quality assessment and acknowledging the importance of mullet (*Mugil platanus*) as an alternative fish resource to substitute the traditional resources the capture and yield of which are declining in Brazil, especially in the Rio de Janeiro State [20], the objective of the present study was to establish chemical parameters to assess the quality of mullet stored on ice (0 ± 1ºC) based on nucleotide degradation and concentration of biogenic amines.

2. Materials and methods

2.1. Samples

A total of 25 mullet specimens with weight ranging from 389.3 to 660.7 grams were acquired, immediately after capture, directly from fishing boats in the Municipality of Maricá (RJ) (22º57'36.1"S, 42º41'48.5"W) from May to August, 2012. The mean ambient water temperature was 23 ± 1ºC. The capture was performed with the aid of nets in hygienic conditions and avoiding fish stress. The specimens were sacrificed by hypothermia in cold water and ice. Then, the fish were transported to the analysis' laboratory in expanded polystyrene isothermal boxes with ice within two hours from the moment of the harvest. There, they were individually washed in tap water and packed in plastic containers with ice (0 ± 1ºC) with a fish/ice ratio of 1:2. The fish were maintained in these conditions for 24 days, period in which the analyses were performed.

2.2. Bacteriological analyses

A 25g aliquot sample was collected on alternate days, under sterile conditions and homogenized with 225 mL of 0.1% Saline Peptone Water (SPW) in a Stomacher (SEWARD – Stomacher 80) type homogenizer to prepare a 10-1 solution. Other dilutions were obtained from this one as the results were obtained. The Aerobic Heterotrophic Mesophilic Bacteria Count (AMHBC) and Psychrotrophic Heterotrophic Bacteria Count (APHBC) were carried out by the methodology described by the American Public Health Association [21] based on the inoculation of 1 mL of the dilutions in plates with Standard Count Agar (SCA). The incubation was carried out in an oven at 35-37ºC for 48 hours for AMHBC and in a refrigerator with temperature controlled at 4 ± 1ºC for seven days for APHBC.

2.3. Determination of nucleotide degradation products

Nucleotide extraction was performed according to the methodology used by Andrade et al. [22]. A 50 mg aliquot was daily collected and homogenized with 1 mL of 8% perchloric acid ($HClO_4$), added of

200 µL 6M potassium hydroxide (KOH) and, after mechanical stirring, centrifuged at 6339 g for 3 minutes. The supernatant was used for compound determination by reverse phase high performance liquid chromatography, according to methodology used by Özogul et al. [23], with some modifications as described below. A Waters® model Alliance® 2695 chromatograph equipped with a Waters® 2996 photodiode array detector, Ultraviolet absorption (UV) at 254 nm, a C_{18} precolumn and a BDS Hypersil C_{18}, 2.4 µm, 100 x 4.6 mm Thermo® column in oven at 30ºC was used. The mobile phase was formed by 0.04 M KH_2PO_4 and 0.06 M K_2HPO_4, pH 7.0 (A) and acetonitrile (B) and a programmed gradient elution with 1.3 $mL.min^{-1}$ flow rate was used. The total time of the chromatographic run was 12 minutes and the analyzed compounds were resolved in 4.5 minutes. After fifteen sample injections the column was flushed in acetonitrile gradient. The adenosine monophosphate (AMP), inosine monophosphate (IMP), inosine (HxR) and hipoxantine (Hx) standards used were from Sigma-Aldrich®.

Based on AMP, IMP, HxR and Hx concentrations, the values Ki [24], G [25] and H [26] were calculated:

$$Ki\ (\%) = (HxR+Hx)/(IMP+HxR+Hx) \times 100$$

$$G\ (\%) = (HxR+Hx)/(AMP+IMP+HxR) \times 100$$

$$H\ (\%) = Hx/(IMP+HxR+Hx\ ) \times 100$$

2.4. Quantification of biogenic amines derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC)

Biogenic amine extraction was conducted according to AOAC methodology [27] with some modifications described below. A 1 g aliquot was daily collected from the sample, homogenized with methanol, placed in a water bath at 60ºC for 15 minutes and then the extract was placed in a 10 mL volumetric flask, adjusting the volume with methanol. The biogenic amines were derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC), which is a reagent that forms more stable fluorescent compounds than those commonly used, and quantified by high performance liquid chromatography according methodology proposed by Cohen and Michaud [28] with some modifications. This reagent forms more stable compounds than those commonly used. The Waters® AccQ•Tag amino acid kit, composed by AQC reagent (Millipore, Milford, MA, EUA) dissolved in acetonitrile and borate buffer solution (200mM boric acid, 5.0mM EDTA, pH 8.8) was used. A 50 µL extract aliquot was dried in a desiccator with activated silica gel. The dry extract was dissolved in 20 µL HCl 20mM solution and 40 µL borate buffer solution, followed by homogenization for 10 and 15 seconds after each reagent addition and derivatized with 20 µL AQC previously prepared. After homogenization the derivatized extract was placed in an oven at 55ºC for 10 minutes and then analyzed by high performance liquid chromatography (Waters® model Alliance® 2695 chromatograph with Waters® 2475 model fluorescence detector, 254nm excitation wavelength and 395nm emission wavelength) using a BDS Hypersil C_{18}, 2.4 µm, 100 x 4.6 mm Thermo® column in oven at 37ºC and mobile phase composed by phase A (sodium acetate solution (60-140mM), triethylamine (5-20mM) and phosphoric acid with pH 5.0-6.5 (AccQ•TagTM) from Waters®) and phase B (acetonitrile) with programmed elution gradient (initial=100%A, 0.5min=99%A, 18min=95%A, 19min=91%A, 29.5min=83%A, 35min=80%A, 36min=78%A, 45min=40%A and 48min=100%A). The flow rate was 1.0 $mL.min^{-1}$ up to 35 minutes of chromatographic run and 1.2 $mL.min^{-1}$ over the remaining 15 minutes. The histamine (HI), putrescine (PU), cadaverine (CA) and tyramine (TI) standards were from Sigma-Aldrich®.

2.5. Statistical analysis

The parameters of bacterial growth (lag phase and generation time) were determined using the DMfit 2.0 computer program based on predictive microbiology and idealized by Baranyi and Roberts [29].

The correlation between the quality indices based on nucleotide concentration with storage time was assessed by the determination of Pearson's coefficient (R). Variance analysis (ANOVA) was used to verify the differences of nucleotide and biogenic amine content among storage times. To help result interpretation, the data obtained (from day 0 to day 24) were grouped into five periods of time, according to Rodrigues et al. [17] proposal: Time 1 (T1) – analysis up to the fourth storage day, time 2 (T2) – analysis from the fifth to the ninth storage day, time 3 (T3) – analysis from the tenth to the 14^{th},

time 4 (T4) – analysis from the 15th to the 19th, and time 5 (T5) – analysis from the 20th to the 24th storage days.

All the analyses were performed using the GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego, California, USA), considering a significance of 5%.

3. Results and Discussion

3.1. Bacteria count

The means of the Aerobic Mesophilic Heterotrophic Bacteria Count (AMHBC) and Aerobic Psychrotrophic Heterotrophic Bacteria Count (APHBC) are represented in **Figure 1**. At storage day 0 (the day of mullet capture) the AMHBC mean was 2.2 log CFU.g^{-1} and APHBC was 1.9 log CFU.g^{-1}. The microbial load of 7.0 log CFU.g^{-1} established by the international commission of microbiologic specifications for food [30] as maximum acceptable level of mesophilic bacteria in fish was attained after the 20th storage day. These results are different from those obtained with other fish species [22,31,32,33,34], however, the initial microbial load and the storage time of this food matrix can be influenced by the fish species involved, bacterial load and type of bacteria present, which can be related to the contamination of the environment and water temperature where the fish lives, and also to the capture, handling and storing conditions [2,5,22,32,33]. The data in **Table 1** shows that the higher adaptation time needed by the mesophilic bacteria indicates that the refrigeration conditions used in the present study favored the quick adaptation of the psychrotrophic bacteria present in the matrix, as reported in previous studies [22,35], as well as the development of this group of bacteria during the storage time as indicated by the smaller generation time of this group of bacteria when compared to mesophilic, which needed 1.6 greater time to multiply than psychrotrophic bacteria. Similar behavior was observed by Ababouch et al. [6] who reported smaller generation time of psychrotrophic bacteria (14.5 hours) than mesophilic bacteria (16.0 hours) in sardine (*Sardina pilchardus*) samples stored in ice. Zaragozá et al [34] and Ensani and Jasour [35] also reported greater load of psycrotrophic bacteria compared to mesophilic bacteria in atlantic salmon (*Salmo salar*) stored at 4ºC and pike-perch (*Sander lucioperca*) stored on ice and frozen storage, respectively.

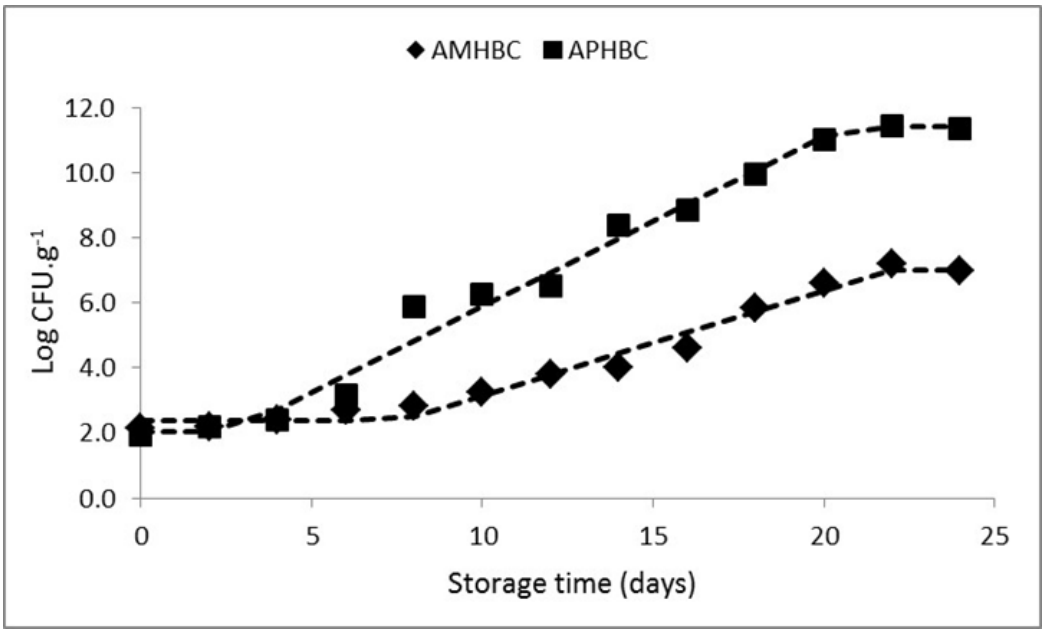

Figure 1: Mean of Aerobic Mesophilic Heterotrophic Bacteria Count (AMHBC) and Aerobic Psychrotrophic Heterotrophic Bacteria Count (APHBC) of mullet (*Mugil platanus*) stored at 0±1ºC for 24 days.

Table 1: Results of Aerobic Mesophilic Heterotrophic Bacteria Count (AMHBC) and Aerobic Psychrotrophic Heterotrophic Bacteria Count (APHBC) growing parameters in mullet (*Mugil platanus*) stored under refrigeration at 0±1ºC for 24 days.

	Initial Count (Log CFU.g^{-1})	Lag (days)	g (hour)	Stationary phase Count (log CFU.g^{-1})	Shelf life (days)
AMHBC	2.2	7.6	22.6	7.2	20
APHBC	1.9	2.7	13.8	11.5	-

Initial Count: Bacteria count in log CFU.g^{-1}; Lag: lag phase in days; g: generation time in hours; Stationary Phase Count: Bacteria count at the beginning of the stationary phase in log CFU.g^{-1}.

3.2. Nucleotide degradation

The content of AMP, IMP, HxR and Hx over the different mullet storage times at 0±1ºC obtained by HPLC is represented in **Figure 2**. The technique employed in this study, through the use of a column with smaller particles (2.4µm) and addition acetonitrile to mobile phase, allowed the resolution of compounds in less time compared to others studies [36,37]. AMP content significantly increased ($p<0.05$) during storage time, from 0.64±0.09 µmol.g^{-1} in time T1 to 0.88±0.20 µmol.g^{-1} in time T5. These results differ from those reported by other researchers that observed AMP content decline during storage time of different fish species [10,38,39,40,41]. However, nucleotide degradation in fish can follow two different paths: one involving the formation of inosine monophosphate and the other that considers a sequence of dephosphorylations to adenosine. In some species both paths can occur. Other species present prevalence of adenosine formation, with consequent AMP accumulation and absence of IMP [42]. In the present study, IMP was detected only up to time T3, because it showed marked decline during the storage time, from 5.15±1.39 µmol.g^{-1} at time T1 to undetectable levels from time T4. The sharp fall of IMP content was also observed in previous studies with different fish species [8,10,11,12,43]. IMP is the main responsible for defining the aroma and flavor of fresh fish [2,5]. Thus, the decline in the concentration of this compound indicates the initial loss of fish quality, in accordance with reports from other authors [8,9,12,13]. HxR content showed marked increase ($p<0.05$) only up to time T3, from 2.94±0.65 µmol.g^{-1} (T1) to 4.60±0.49 µmol.g^{-1} (T3). After this time, the concentration of this compound signficanty attaining 3.25±0.47 µmol.g^{-1} at time T5. A similar behavior wasobserved by

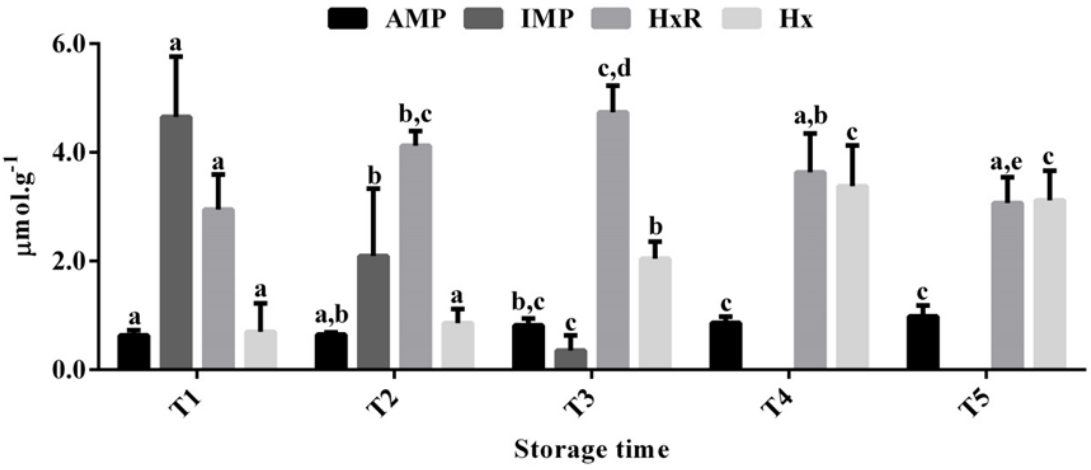

Figure 2: Concentrations of adenosine monophosphate (AMP), inosine monophosphate (IMP), inosine (HxR) and hypoxanthine (Hx) in mullet (*Mugil platanus*) kept at 0±1ºC for different storage times: T1 (0 to 4th storage day), T2 (5th to 9th storage day), T3 (10th to 14th storage day), T4 (15th to 10th and T5 (20st to 24th storage days). Mean concentrations (n = 8). Standard deviations are indicated by bars. Different letters indicates the statistically significant differences of values between storage time ($p<0.05$).

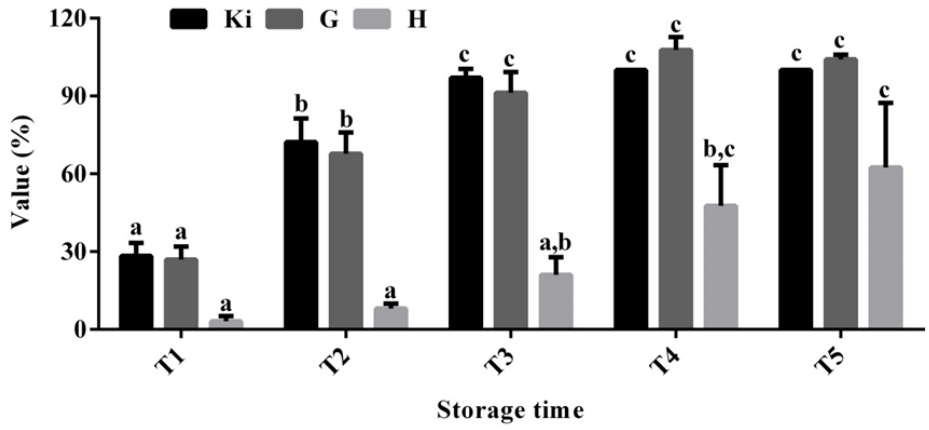

Figure 3: Mean Ki, G and H values in mullet (*Mugil platanus*) kept at 0±1ºC for different storage times: T1 (0 to 4th storage day), T2 (5th to 9th storage day), T3 (10th to 14th storage day), T4 (15th to 10th and T5 (20st to 24th storage days). Mean concentrations (n = 8). Standard deviations are indicated by bars. Different letters indicates the statistically significant differences of values between storage time ($p<0.05$).

otherauthors in previous studies with different fish species [9,11,13,44]. Hx content at time T1 was 0.51±0.53 µmol.g^{-1} and significantly increased during storage time mainly at times T3 (2.17±0.32 µmol.g^{-1}) and T4 (3.20±0.75 µmol.g^{-1}). Hx accumulation reflects the initial phase of autolytic spoilage, as well as bacterial spoilage and thus is an important quality parameter for different fish species, as observed in the present study and reported in previous ones [9,10,13,22]. Some fish species accumulate HxR from IMP while others accumulate Hx. There are also species that accumulate both HxR and Hx, however Hx accumulation occurs slower [45,46,47]. HxR is relatively tasteless while Hx has a direct effect on the bitter flavor of spoiled fish [2,5]. In the present study, HxR mean concentration was higher than that of Hx up to time T4, however from time T3, when IMP was not detected anymore, as HxR content declined, there was a significant increase of Hx content. Thus, in the same way that IMP and HxR decline, Hx accumulation can also be used as a measure of mullet quality loss.

The values Ki, G and H calculated from AMP, IMP, HxR and Hx concentration in mullet samples stored in ice (0±1ºC) are represented in **Figure 3**. These values have been proposed as quality index due the differences that species present in relation to mechanisms of degradation of nucleotides [8,24,25,26]. Ki value presented good correlation (R=0.88) with storage time, however, marked increase occurred only up to T3 (T1~28%, T2~74% and T3~97%). From T4, IMP was not detected and consequently Ki value was constant (T4=T5~100%). Ki value was suggested by Karube et al. [24] to replace K [48] and does not take into consideration the intermediary products of ATP degradation to IMP, because in some species this process occurs very fast impairing the identification of those compounds. G value showed high correlation (R=0.92) with storage time with a significant increase ($p<0.05$) during the initial storage times (T1~26%, T2~69% and T3~90%). FromT4 its increase was not significant ($p>0.05$), reaching at time T5~104%. G value, according to Burns et al. [25] is based in Hx accumulation and IMP degradation, however the rapid increase observed in both this value and the Ki value during the three initial storage times is due not only to the progressive Hx increase and the rapid degradation of IMP, but also to HxR content gradual increase, as was also observed by other authors [9,11]. H was the value that presented the best correlation (R=0.97) with storage time. As this value is based solely on Hx concentration [11,26,49], a significant increase ($p<0.05$) was observed only from time T3, when high Hx levels were also detected. In time T1 H value was approximately 3% reaching about 57% in time T5.

3.3. Biogenic amines

The content of the biogenic amines derivatizated with AQC researched by HPLC in mullet stored on ice (0±1ºC) is shown in **Figure 4**. The use of the AQC as derivatizing reagent allowed of forming stable compounds, as reported in others studies [28,50,51]. Histamine content did not significantly increasewi-

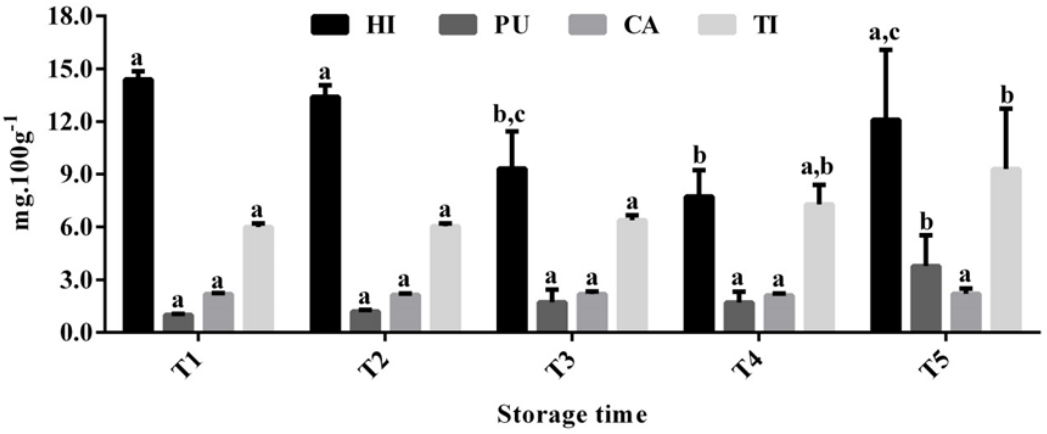

Figure 4: Mean of histamine (HI), putrescine (PU), cadaverine (CA) and tyramine (TI) concentrations in mullet (*Mugil platanus*) kept at 0±1ºC for different storage times: T1 (0 to 4th storage day), T2 (5th to 9th storage day), T3 (10th to 14th storage day), T4 (15th to 10th and T5 (20st to 24th storage days). Mean concentrations (n = 8). Standard deviations are indicated by bars. Different letters indicates the statistically significant differences of values between storage time ($p<0.05$).

thstorage time, fact related to amino acid substrate. The variation of biogenic amine content in fish may be a function of feeding, sex, physiological stage, tissue (sample aliquot), among other factors [6,14,52], which explains histamine behavior observed during storage of the samples in the present study. In this manner, although histamine was the amine found in the highest concentration in the samples (7.84 to 13.4 mg.100g^{-1}), it is not adequate for quality assessment of mullet kept under those storage conditions. The presence of this biogenic amine is not necessarily related to fish spoilage, as reported in studies of several fish species [9,10,15,53]. Cadaverine was also considered not adequate for mullet quality assessment, because its concentration was constant during the entire storage period (2.08 to 2.19 mg.100g^{-1}). However the research theses amines is fish has high importance because the histamine is often associated with allergic poisoning and the cadaverine is able to potentiate the action of histamine and form carcinogenic compounds [18,19,54,55]. Putrescine and tyramine contents were constant until the last storage time (T5), when they increased from 1.01±0.06 mg.100g^{-1} and 5.89±0.21 mg.100g^{-1} at time T1, to 3.42±1.74 mg.100g^{-1} and 8.41±3.44 mg.100g^{-1} at time T5, respectively. The significant increase of theses amines just in time T5, corresponded to the day when mullet was microbiologically rejected, because it attained the limit of 7 log CFU.g^{-1} for mesophilic count in fish under refrigeration [30], which was the 20th storage day. Thus, the biogenic amines putrescine and tyramine have high potential for being used as quality parameters in the assessment of mullet stored in ice. The potential of these amines as quality indicators was also observed for other fish species, associated to other biogenic amines or individually [14,15,16,17,53].

3. Conclusion

The nucleotides IMP, HxR and Hx and the biogenic amines PU and TI are potential compounds for use as chemical parameters to assess the quality of mullet stored at 0±1ºC. The IMP and Hx showed high correlation with quality loss of mullet, as well as the PU and TI, while HxR proved to be adequate to evaluate mullet freshness during the first days of storage. The results obtained show that mullet can be consumed up to the 20th storage day, when obtained in similar conditions as in the present study and kept at the same storage conditions (0±1ºC).

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3.2 ARTIGO 2: CHEMICAL STABILITY OF WHITEMOUTH CROAKER (*Micropogonias furnieri*) STORED AT 0 AND 5ºC. Submitted to Food Chemistry.

Chemical stability of whitemouth croaker (*Micropogonias furnieri*) stored at 0 and 5 ºC

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ABSTRACT

The effect of storage temperature on quality parameters of whitemouth croaker (*Micropogonias furnieri*) was investigated determining nucleotide breakdown products by HPLC-PDA; Ki, G and H levels; biogenic amines by HPLC-FLD; mesophilic and psychrotrophic bacteria counts. The bacterial groups multiplied twice as fast in samples stored at 5±1ºC compared to samples kept at 0±1ºC, reducing shelf life by 10 days. IMP levels rapidly declined, while Hx increased with storage time. IMP and Hx concentrations were five times lower and two times higher, respectively, in samples stored at 5±1ºC. Pearson coefficients (R) for Ki, G and H were 0.99, 0.98 and 0.98, respectively for samples stored at 0±1ºC, and R=0.99 for all values calculated for samples stored at 5±1ºC. Cadaverine and

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putrescine concentrations were influenced by the storage time. Histamine and tyramine levels were significantly higherat 5±1ºC. IMP and Hx, histamine and tyramine levels are potential quality indicators for freshness evaluation**.**

Keywords: biogenic amines, nucleotide, quality parameters, refrigeration temperature, histamine, hypoxanthine.

1. Introduction

The whitemouth croaker (*Micropogonias furnieri*) is a coastal species of high commercial interest in southern and southeastern Brazil, Uruguay and Argentina (Borges et al., 2007; Teixeira, Borges, Franco, São Clemente & Freitas, 2009). In Brazil in 2011, this species was the second most frequently caught, comprising about 3.0% of total production (BRASIL, 2013). Fish for direct human consumption are largely sold live, fresh or chilled (FAO, 2012; BRASIL, 2013). However, fresh fish are highly perishable due to their chemical composition, *post-mortem* pH close to neutrality, high water activity, and content of easily oxidized polyunsaturated fatty acids (Gram & Huss, 1996; Rodrigues et al., 2013). Thus, the quality of fresh fish is mainly influenced by the fishing conditions and maintenance of storage temperatures (Ababouch, Souibri, Rhaliby, Ouahdi, Battal & Busba, 1996; Lakshmanan, Antony & Gopakumar, 1997; Özogul, Özogul & Kuley, 2008; Song, Luo, You, Shen & Hu, 2012).

After a fish is caught and dies, a series of chemical and microbiological reactions begins, with the formation of compounds that are often used to assess the quality of its flesh (Gram & Huss, 1996). Adenosine triphosphate (ATP) degradation may reflect the earliest changes in fish immediately after death. ATP, by the action of enzymes present in muscle, is

dephosphorylated to adenosine diphosphate (ADP), and adenosine monophosphate is deaminated to inosine monophosphate (IMP) which degrades into inosine (HxR) and hypoxanthine (Hx) (Song et al., 2012). Thus, the nucleotide level has often been used as a quality indicator (Özogul, Özogul & Gökbulut, 2006; Özogul et al., 2008; Song et al., 2012).

The concentration of biogenic amines, individually or together, also has been widely proposed as a quality indicator for fish (Veciana-Nógues, Mariné-Font & Vidal-Carou, 1997; Křížek, Pavlíček & Vácha, 2002; Cunha et al., 2013; Rodrigues et al., 2013). These organic compounds of basic character and low molecular weight are formed mainly by decarboxylation of precursor amino acids (Silva, Fernandez, Fonseca, Mársico & São Clemente, 2011; Silva, Lázaro, Mársico, Mano & Conte-Junior, 2013; Rodriguez, Carneiro, Feijó, Conte Júnior & Mano, 2014) and, when present in high amounts in fish products, may be associated with improper storage with subsequent microbial decarboxylation of amino acids (Hu, Huang, Li & Yang, 2012; Rodriguez et al., 2014).

High-performance liquid chromatography is the technique of choice for the determination of nucleotides and biogenic amines (Özogul et al., 2008; Özogul, Boğa, Tokur & Özogul, 2011; Lázaro et al., 2013; Önal, Tekkeli & Önal, 2013; Ordóñez, Callejón, Morales & García-Parrilla, 2013). However, the optimization of preparative steps and chromatographic conditions to allow rapid detection with low cost and high sensitivity is essential for the quality control in the food industry and the scientific community.

There is a need to identify chemical quality markers and appropriate analytical techniques that provide concrete data for this food. The aim of this study was to determine the contents of AMP, IM, HxR and Hx and the levels of histamine, putrescine, cadaverine and tyramine in whitemouth croaker (*Micropogonias furnieri*) by high-performance liquid chromatography, and to investigate the influence of temperature (0 ± 1 and 5 ± 1 ºC) and storage time on quality chemical parameters based on the concentrations of these compounds.

2. Material and Methods

2.1. Fish

Fifty whitemouth croaker (*Micropogonias furnieri*) weighing between 413.0 and 822.8 grams were purchased from fishing boats in the municipality of Maricá, the lake region of the state of Rio de Janeiro, Brazil (22º57'36.1"S, 42º41'48.5"W) from May to August 2012. Immediately after capture, the fish were killed by immersion in ice-water slurry. After death, fish were placed in expanded-polystyrene coolers with ice and transported to the physico-chemical analysis laboratory within two hours. In the laboratory, the fish were individually rinsed and divided into two groups. One group of 25 was placed in plastic containers with ice (0±1 ºC) and analyzed for 20 days. The other group, from the same sample, was placed in plastic containers and stored in a refrigerator (5±1 ºC), and analyzed for 15 days. Ten fish kept in different storage conditions (0±1 or 5±1 ºC) were used for bacteriological analysis, performed on alternate days. The remaining 15 fish stored at 0±1 or 5±1 ºC were used for daily determinations of the nucleotide and biogenic amines.

2.2. Bacteriological analysis

The bacterial counts were performed every other day in samples that were separated for this purpose but stored under the same conditions as the other samples. A sample of 25 g of each specimen was collected under sterile conditions, homogenized with 225 mL of 0.1% saline peptone water by a stomacher (SEWARD – Stomacher 80), and diluted by a factor of 10, from which other dilutions were produced. The aerobic mesophilic heterotrophic and psychrotrophic bacteria (AMHB and AMPB, respectively) were counted as recommended by

the American Public Health Association (APHA, 2001), which requires spreading 1 mL of the different dilutions on standard plate count agar (PCA). AMHB were incubated at 35-37 ºC for 48 h and AMPB at 4±1 ºC for seven days.

2.3 Determination of nucleotides

Nucleotide extraction was carried out according to the methodology used by Andrade et al. (2012). Fifty milligrams of fish without skin was hand-chopped and extracted with 1 mL of 8% perchloric acid ($HCLO_4$), using ultrasound, for 10 min. The extraction mixture was neutralized with 200 µL of 6 M potassium hydroxide (KOH) and homogenized with mechanical stirring for 20 s. In the next step, the mixture was centrifuged at 6300.0 g for 3 min and the supernatant was transferred to the vial with the aid of an automatic pipette.

Reverse-phase high-performance liquid chromatography was carried out according to Özogul, Taylor, Quantick and Özogul (2000) with modifications, using a Waters® model Alliance® 2695 chromatograph equipped with a Waters® 2996 photodiode array detector, a C_{18} pre-column and a BDS Hypersil™ C_{18}, 2.4 µm 100 x 4.6 mm Thermo® column in an oven at 30ºC. The mobile phase A was a buffer solution of pH 7.0 prepared with 0.04 M KH_2PO_4 and 0.06 M K_2HPO_4 dissolved in purified distilled water, and phase B was HPLC grade acetonitrile. The programmed gradient elution with a flow rate of 1.3 $mL.min^{-1}$ was as follows: 0 min, 100% A and 0% B; 2.1 min, 95% A and 5% B; 5 min, 75% A and 25% B; 5.1 min, 100% A and 0% B. The acetonitrile was added to decrease the retention time of the compounds analyzed. The injection volume was 5 µL, and detection was monitored by Ultraviolet absorption (UV) at 254 nm. After 15 samples were injected, the column was washed with an acetonitrile gradient to remove any material retained.

Nucleotide standards (Adenosine 5'-Triphosphate, Adenosine 5'-Diphosphate, Adenosine 5'-Monophosphate, Inosine 5'-Monophosphate, Inosine, Hypoxanthine) were purchased from Sigma-Aldrich®.

2.4. Quality index

The Ki, G and H values were calculated by the procedures described by Karube, Matsuoka, Suzuki, Watanabe and Toyama (1984), Burns, Ke and Irvine (1985) and Luong, Male, Masson and Nguyen (1992), respectively. The formulas are as follows:

$$Ki\ (\%) = \frac{HxR + Hx}{IMP + HxR + Hx}\ x\ 100$$

$$G(\%) = \frac{Hx + HxR}{HxR + IMP + AMP}\ x\ 100$$

$$H(\%) = \frac{Hx}{IMP + Hx + \ HxR}\ x\ 100$$

2.5 Quantification of biogenic amines derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC)

Biogenic amines were extracted according to AOAC methodology (2002), with modifications. One gram of fish without skin was hand-chopped and extracted with methanol under mechanical stirring for 20 s and placed in a water bath at 60 ºC for 15 min. In the next

step the extraction mixture was filtered on qualitative filter paper into a 10 mL volumetric flask, and the volume adjusted with methanol.

Biogenic amines derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) were analyzed by high-performance liquid chromatography according to Cohen and Michaud (1993), with modifications. Pre-column derivatization of biogenic amines with AQC was performed using Waters® AccQ•Tag® amino acid kit composed of AQC reagent (Millipore, Milford, MA, USA) dissolved in acetonitrile and borate buffer (200 mM boric acid, 5.0 mM EDTA, pH 8.8). Fifty microliters of the extract was transferred to the vial and placed in a desiccator with activated silica gel for drying for a mean of 24 h. The dry extract was dissolved in 20 µL HCl 20 mM solution and 40 µL borate buffer solution, followed by homogenization for 10 to 15 s after each reagent addition, and derivatized with 20 µL AQC reagent previously prepared. After a 1-min rest, the mixture was heated at 55 ºC for 10 min to finalize the derivatization reaction. Derivatized samples were transferred to a vial. The same procedure was used for derivatization of 10 µL of each standard solution. The chromatographic separation of biogenic amines was performed using a Waters® model Alliance® 2695 chromatograph equipped with a Waters 2475 fluorescence detector, 254 nm excitation wavelength and 395 nm emission wavelength, and a BDS Hypersil C_{18}, 2.4 µm, 100 x 4.6 mm Thermo® column in an oven at 37 ºC. Mobile phase A consisted of sodium acetate (60-140 mM), triethylamine (5-20 mM) and phosphoric acid with pH 5.0-6.5 (Eluent A AccQ•Tag™ Waters®); phase B was HPLC-grade acetonitrile. The gradient elution was programmed (initial=100%A, 0.5min=99%A, 18min=95%A, 19min=91%A, 29.5min=83%A, 35min=80%A, 36min=78%A, 45min=40%A and 48min=100%A). The flow rate was 1.0 mL.min-1 up to 35 minutes of chromatographic run and 1.2 mL.min-1 over the remaining 15 minutes. The injection volume was 10 µL.

Standards of biogenic amines (histamine, putrescine, cadaverine and tyramine) were purchased from Sigma-Aldrich®.

2.7 Statistical analysis

The parameters of bacterial growth (lag phase and generation time) were determined by the software DMfit 2.0 based on predictive microbiology and idealized by Baranyi and Roberts (1994).

The correlation between the quality indices (Ki, G and H values) and the storage time was determined using Pearson's coefficient (R). Variance analysis (ANOVA) was used to assess differences in the levels of nucleotide and biogenic amines between temperatures and storage times. The data obtained for each storage temperature (0±1 ºC and 5±1 ºC) were divided into periods, as suggested by Rodrigues et al. (2013). The results obtained from days 0 to 5 of storage were grouped at time 1 (T1), from days 6 to 10 at time 2 (T2), and from days 11 to 15 at time 3 (T3). For the samples stored at 0±1 ºC, time 4 (T4) refers to the results for days 16 to 20 of storage. All analyses were done with GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego, CA, USA), using a significance level of 5%.

3. Results and Discussion

3.1 Bacterial counts

The means of Aerobic Mesophilic Heterotrophic Bacteria (AMHB) and Aerobic Psychrotrophic Heterotrophic Bacteria (APHB) counts in whitemouth croaker kept in different temperatures are shown in Fig. 1. The initial load of mesophilic and psychrotrophic

bacteria was 2.3 log CFU.g^{-1} and 2.2 log CFU.g^{-1}, respectively. The AMHB counts obtained here were lower than in previous studies (Montagner et al., 2005; Teixeira et al., 2009), possibly due to the superior quality of the raw material used in the present study, which is related to appropriate catch conditions and maintenance of the variables in the storage steps that determine the shelf life. The International Commission of Microbiological Specifications for Foods (ICMSF, 1986) recommends a count of 7.0 log CFU.g^{-1} for mesophilic bacteria in chilled fish as the maximum acceptable limit. This value was reached after the 16th and 6th storage days of the whitemouth croaker at 0±1 and 5±1 ºC, respectively. Teixeira et al. (2009) reported that for whitemouth croaker kept on ice, this limit was reached on the 14th storage day, a shorter time than in the present study, for the samples kept under the same temperature conditions. This difference may be related to the higher initial microbial load observed by these authors, or to possible differences in conditions on the fishing boats.

The lag phase (lag) of the APHB was shorter than that observed for AMHB, regardless of storage temperature. The longer time required by mesophiles to adapt indicates that refrigeration favored the development of psychrotrophic bacteria present in the matrix, which quickly adapted to the storage conditions. Similarly, this bacterial group multiplied rapidly, which multiplied over a 1.8 times longer period compared to the time observed in the psychrotrophic group.

The storage temperature of the fish influences bacterial growth and consequently the degradation rate of this food (Ababouch et al., 1996; Chong, Bakar, Rahman, Bakar & Zaman, 2012; Song et al., 2012). Besides the shorter adaptation time of mesophilc and psychrotrophic bacteria in samples kept at 5±1 ºC, these bacterial groups grew faster in samples kept under these conditions (10.7 and 5.8 h, respectively) compared to samples stored at 0±1 ºC (23.9 and 13.4 h, respectively), which represented a decrease of ten days of shelf life of whitemouth croaker kept at the higher temperature. As reported in previous studies

(Ababouch et al., 1996; Veciana-Nógues et al., 1997; Olafsdottir, Lauzon, Martinsdottir & Kristbergsson, 2006), the shelf life of fish may decrease at higher storage temperatures.

3.2 Nucleotide degradation products

The chromatogram of the nucleotide degradation products in a sample of whitemouth croaker is shown in Fig. 2 The chromatographic run lasted for 13 min and allowed the simultaneous determination in 4.5 min. The use of a column with smaller particles (2.4 µm) than those used in previous studies (Lakshmanan et al., 1996; Veciana-Nógues et al., 1997; Özogul et al., 2000; Özogul et al., 2011) reduced the retention time of the compounds and improved the resolution of the peaks. Additionally, the use of the organic solvent acetonitrile, as proposed by Özogul et al. (2000), also helped to reduce the retention time of the substances.

AMP, IMP, HxR and Hx contents in the whitemouth croaker kept at 0±1 ºC and 5±1 ºC for different storage times are shown in Fig. 3. Nucleotide degradation was more rapid in samples stored at 5±1 ºC than on ice (0±1 ºC). AMP levels were low (~0.25 to 0.39 µmol.g^{-1}); however, as the ATP hydrolysis to IMP occurs rapidly by endogenous enzymes (Gram & Huss, 1996), usually low AMP levels are detected, as also observed in studies with different fish species (Özogul et al., 2011; Song et al., 2012; Li, Li & Hu, 2013; Rzepka, Özogul, Surówka & Michalczyk, 2013). The AMP level increased significantly ($p<0.05$) in the second storage time (T2) for the samples kept at 0±1 ºC, but decreased at time T3 and remained constant until time T4. No significant difference ($p>0.05$) was observed in the AMP levels in the samples stored at 5±1 ºC during the storage period. IMP was most prominent in the early storage period (9.81±3.85 µmol.g^{-1} and 8.03±3.15 µmol.g^{-1}, in the samples kept at 0±1 and 5±1 ºC, respectively). In agreement with observations in previous studies (Aubourg et al.,

2007; Lakshmanan et al., 1996; Özogul et al., 2011; Song et al., 2012; Li et al., 2013) the IMP level decreased rapidly during the storage period, mainly in the samples stored at 5±1 ºC, reaching 0.68±0.67 μmol.g^{-1} at time T3. The storage temperature significantly ($p<0.05$) influenced the IMP level from time T2, reaching 6.64±1.70 μmol.g^{-1} at time T3 for samples stored at 0±1 ºC, a level nine times higher than those for samples stored at 5±1 ºC in the same period (T3). IMP accumulates in the initial stage of degradation of fish and is responsible for defining the flavor of fresh fish (Gram & Huss, 1996). Thus, the decline in the concentration of this compound is indicative of an initial loss of quality, as reported by other authors (Aubourg et al., 2007; Özogul et al., 2008; Li et al., 2013; Rzepka et al., 2013). The HxR content increased gradually over the storage period for the samples kept at 0±1 ºC, mainly from time T3, when the level of this compound was significantly higher ($p<0.05$) than that obtained in the samples stored at 5±1 ºC. At this storage temperature, the HxR content increased significantly ($p<0.05$) only from time T2 and then decreased. In white grouper (*E. aeneus*), Özogul et al. (2008) also observed a decrease in the HxR concentration after the eighth day of ice storage. Similar behavior was observed by Song et al. (2012) and Rzepka et al. (2013) in bream (*Megalobrama amblycephala*) kept at 4.5 ºC and in gravid Atlantic bonito (*Sarda sarda*) stored at 7±1 ºC, respectively. There was Hx accumulation during storage of the whitemouth croaker, as reported in previous studies with other fish species (Özogul et al., 2008; Özogul et al., 2011; Andrade et al., 2012; Rzepka et al., 2013). The Hx level in the samples kept at 0±1 and 5±1 ºC was similar at time T1 (2.60±0.23 μmol.g^{-1} and 2.16±0.33 μmol.g^{-1}, respectively), however from time T2, Hx level increased significantly in the samples kept at 5±1ºC, reaching 7.09±0.36 μmol.g^{-1} at time T3, a level two times higher than in samples stored at 0±1 ºC for the same time (T3) (3.17±0.46 μmol.g^{-1}). Hx is formed by endogenous and microbial enzymes and has a direct effect on the bitter taste of spoiled fish (Gram & Huss, 1996), which makes it a suitable parameter indicative of quality loss, as

reported by other authors (Aubourg et al., 2007; Özogul et al., 2011; Li et al., 2013; Rzepka et al., 2013).

The Ki, G and H values of whitemouth croaker stored at 0±1 ºC and 5±1 ºC are shown in Fig. 4. Linear increases were observed in the Ki, G and H values in the samples stored at 0±1 ºC (R=0.99, R=0.98 and R=0.98, respectively) and in the samples kept at 5±1 ºC (R=0.99), as also reported in other studies (Özogul et al., 2006; Özogul et al., 2008; Song et al., 2012). The Ki value remained statistically constant only between T2 and T3, but increased rapidly in samples stored at 5±1 ºC (T1~39% and T3~94%) and in the samples kept at 0±1 ºC (T1~37% and T4~74%). The increase in the G was significant ($p<0.05$) between all storage times. This value is based on the Hx accumulation and IMP degradation (Burns et al., 1985; Özogul et al., 2008; Song et al., 2012), which explains the significant increase, mainly in samples stored at 5±1 ºC, as well as the high values found (approximately 43% and 45% at time T1 and 101% and 228% at time T4 and T3 in the samples kept at 0±1 ºC and 5±1 ºC, respectively). The significant increase ($p<0.05$) of the H value occurred between all storage times in the samples kept at 5±1 ºC (T1~16%, T2~34% and T3~61%), and only at time T4 (T1~17%, T2~19%, T3~22% and T4~29%) of the samples stored at 0±1 ºC. The H value is based only on the Hx concentration (Luong et al., 1992; Song et al., 2012), which explains the higher values in the samples stored at 5±1 ºC.

3.4. Biogenic amines

The derivatization reactions of biogenic amines occur via the amino group and various types of tagging reagents, such as *o*-phthaldialdehyde (OPA), dansyl chloride (dansyl-Cl), 4-chloro-3,5-dinitrobenzotrifluoride (CNBF), 1,2-naphthoquinone-4-sulfonate (NQS), 6-aminoquinolyl-*N*-hydroxysuccinimidyl (AQC), and *N*-hydroxy-succinimide ester (DMQC-

Osu). AQC, which reacts with primary and secondary amines, forms more stable fluorescent compounds than the others (Fiechter, Sivec & Mayer, 2013; Ordóñez et al., 2013). The pre-column derivatization with AQC developed by Cohen and Michaud (1993) for chromatographic analysis of amino acids has been adapted for the analysis of biogenic amines in various food matrices (Fiechter et al., 2013; Ordóñez et al., 2013). However, reports of the use of this reagent for analysis of biogenic amines in fish are few.

In the chromatogram of the biogenic amines in samples of whitemouth croaker (Fig. 5), the presence of several peaks of amino acids did not affect the analysis, and it was possible to identify the biogenic amines by retention time. In future studies, a prior sample treatment such as solid-phase extraction is recommended.

Levels of histamine, putrescine, cadaverine and tyramine in the whitemouth croaker kept at 0±1 ºC and 5±1 ºC for different storage times are shown in Fig. 6. Putrescine and cadaverine levels were not influenced significantly ($p>0.05$) by the storage temperature, but increased significantly ($p<0.05$) with storage time. In the samples kept at 0±1 ºC, the putrescine level increased significantly ($p<0.05$) only from time T3, reaching 0.24±0.10 $mg.100g^{-1}$ in the last time period (T4). In the samples stored at 5±1 ºC, no significant difference ($p>0.05$) in the putrescine content was observed (0.10±0.02 $mg.100g^{-1}$ at time T1 and 0.15±0.05 $mg.100g^{-1}$ at time T3). Cadaverine level increased significantly ($p<0.05$) only from time T3 in the samples kept at the two temperatures, 0.28±0.08 $mg.100g^{-1}$ and 0.20±0.03 $mg.100g^{-1}$ at time T4 and T3 for the samples kept at 0±1 ºC and 5±1 ºC, respectively. These results differ from those obtained by Rodrigues et al. (2013) and Křížek et al. (2002); however, the type and content of biogenic amines in fish can vary according to the species (Ababouch et al., 1996; Veciana-Nógues et al., 1997). In the present study, although putrescine and cadaverine did not prove to be appropriate quality indicators for whitemouth croaker, they are important amines because they potentiate the action of histamine and are

precursors of carcinogenic compounds, forming nitrosamines (Rzepka et al., 2013). The histamine concentration increased significantly ($p<0.05$) during the storage period, as also reported by Borges et al. (2007). In the samples kept at 0±1 ºC, histamine increased from 1.24±0.20 mg.100g^{-1} at time T1 to 1.94±0.45 mg.100g^{-1} at time T4. The storage temperature influenced the increased level of this amine at time T3, reaching 2.19±0.67 mg.100g^{-1} in samples stored at 5±1 ºC, a significantly higher concentration ($p<0.05$) than observed in samples kept at 0±1 ºC (1.50±0.18 mg.100g^{-1}) for the same period. Whitemouth croaker, as reported by Izquierdo et al. (2001), has a high concentration of free histidine in its muscle. Histidine can be decarboxylated to histamine (Silva et al, 2011; Rodriguez et al., 2014), which explains the high histamine level obtained in this study, and makes this amine an important quality indicator for this species, similarly to findings with other fish species (Veciana-Nógues et al., 1997; Hu et al., 2012). The importance of this amine in fish, besides its high correlation with the quality of these products, is its potential toxicological risk, often associated with allergic poisoning characterized by urticaria, headache and decrease of blood pressure (Silva et al., 2011; Rodriguez et al., 2014). Tyramine was most influenced by storage time and temperature. At time T1 the concentrations of this amine were similar in the two storage temperatures (0.57±0.01 mg.100g^{-1} and 0.59±0.05 mg.100g^{-1}, in the samples stored at 0±1 ºC and 5±1 ºC, respectively). However, at time T2, the concentration of this amine increased significantly ($p<0.05$) in the samples kept at 5±1 ºC (1.60±1.50 mg.100g^{-1}). In samples stored at 0±1 ºC, a significant increase ($p<0.05$) occurred only at time T4 (2.83±2.23 mg.100g^{-1}). Tyramine is formed from the amino acid tyrosine, and is known for its sympathomimetic properties, often associated with hypertensive crises and migraine (Silva et al., 2011; Rodriguez et al., 2014). Among the essential amino acids found in whitemouth croaker, Izquierdo et al. (2001) reported that tyrosine was the most abundant, which explains the high tyramine content found in this species in the present study. Thus, tyramine has a high

potential for quality assessment of the whitemouth croaker, as observed in studies with other fish species (Veciana-Nógues et al., 1997; Hu et al., 2012).

4. Conclusions

The temperature of 5±1 ºC decreased the lag phase and generation time of microorganisms present in whitemouth croaker, shortening the shelf life of this product by ten days compared to samples kept at 0±1 ºC. IMP and Hx levels can be used as an early indicator of quality loss in whitemouth croaker. The quality indexes based on the concentration of nucleotides were highly correlated with the storage time. The behavior of the histamine and tyramine levels showed that these amines have good potential for use as quality indicators in the assessment of whitemouth croaker.

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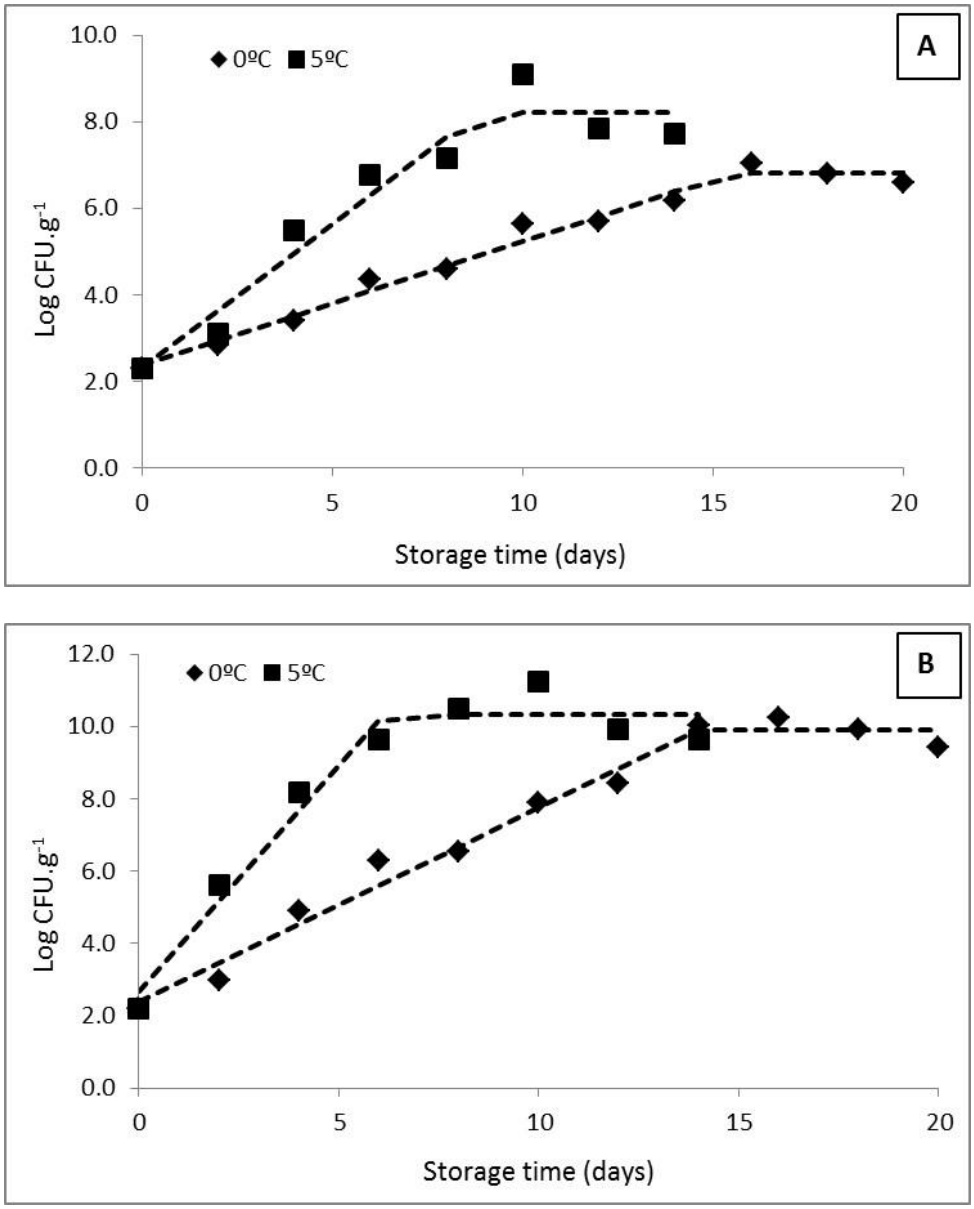

Fig. 1: Aerobic mesophilic heterotrophic (AMHBC) (A) and psychrotrophic (AMPBC) (B) bacterial counts in whitemouth croaker (*Micropogonias furnieri*) kept at 0±1 ºC or 5±1 ºC.

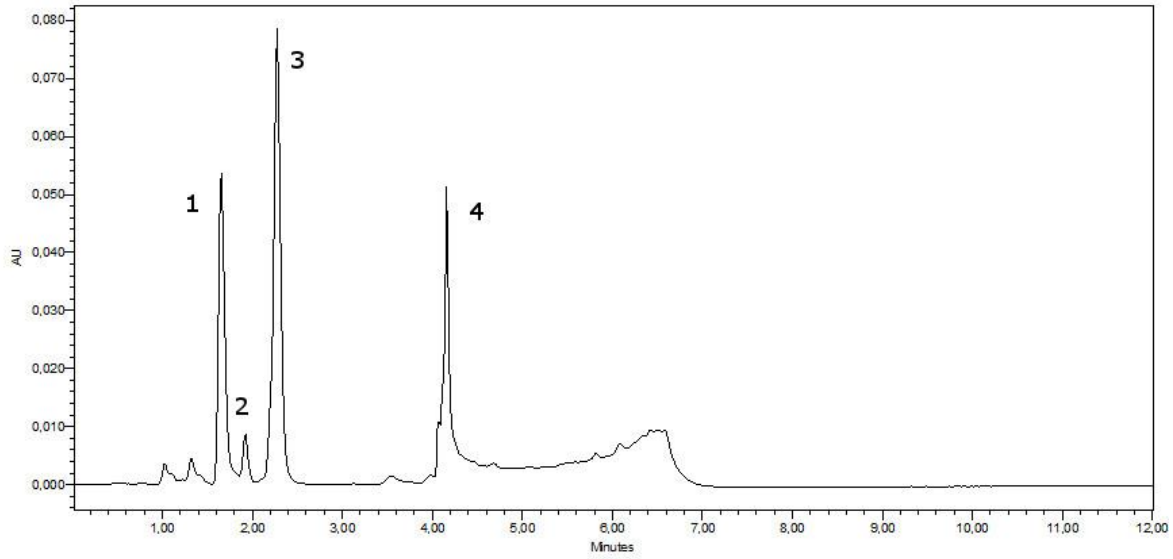

Fig. 2: Typical chromatogram for separation of nucleotides in refrigerated samples of whitemouth croaker (*Micropogonias furnieri*): 1, inosine monophosphate; 2, adenosine monophosphate; 3, hypoxanthine; 4, inosine.

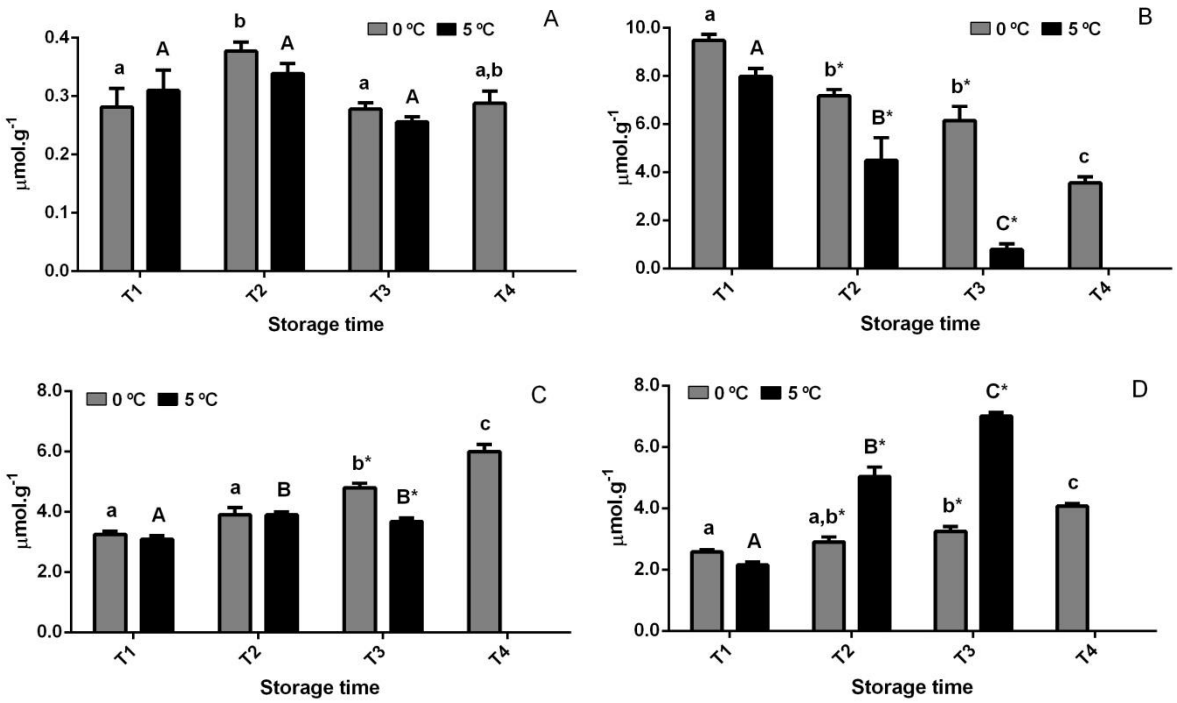

Fig. 3: Levels of the nucleotides adenosine monophosphate (A), inosine monophosphate (B), inosine (C) and hypoxanthine (D) in whitemouth croaker (*Micropogonias furnieri*) kept at 0±1 ºC or 5±1 ºC for different storage times: T1 (0 to 5th storage day), T2 (6th to 10th storage day), T3 (11th to 15th storage day), T4 (16th to 20th storage days). Mean levels (n = 8). Standard deviations are indicated by bars. Different lowercase and capital letters indicate significant differences between values ($p<0.05$) in the samples stored at 0±1 and 5±1 ºC, respectively. * Values with significant differences ($p<0.05$) between the storage temperatures, at the same storage time.

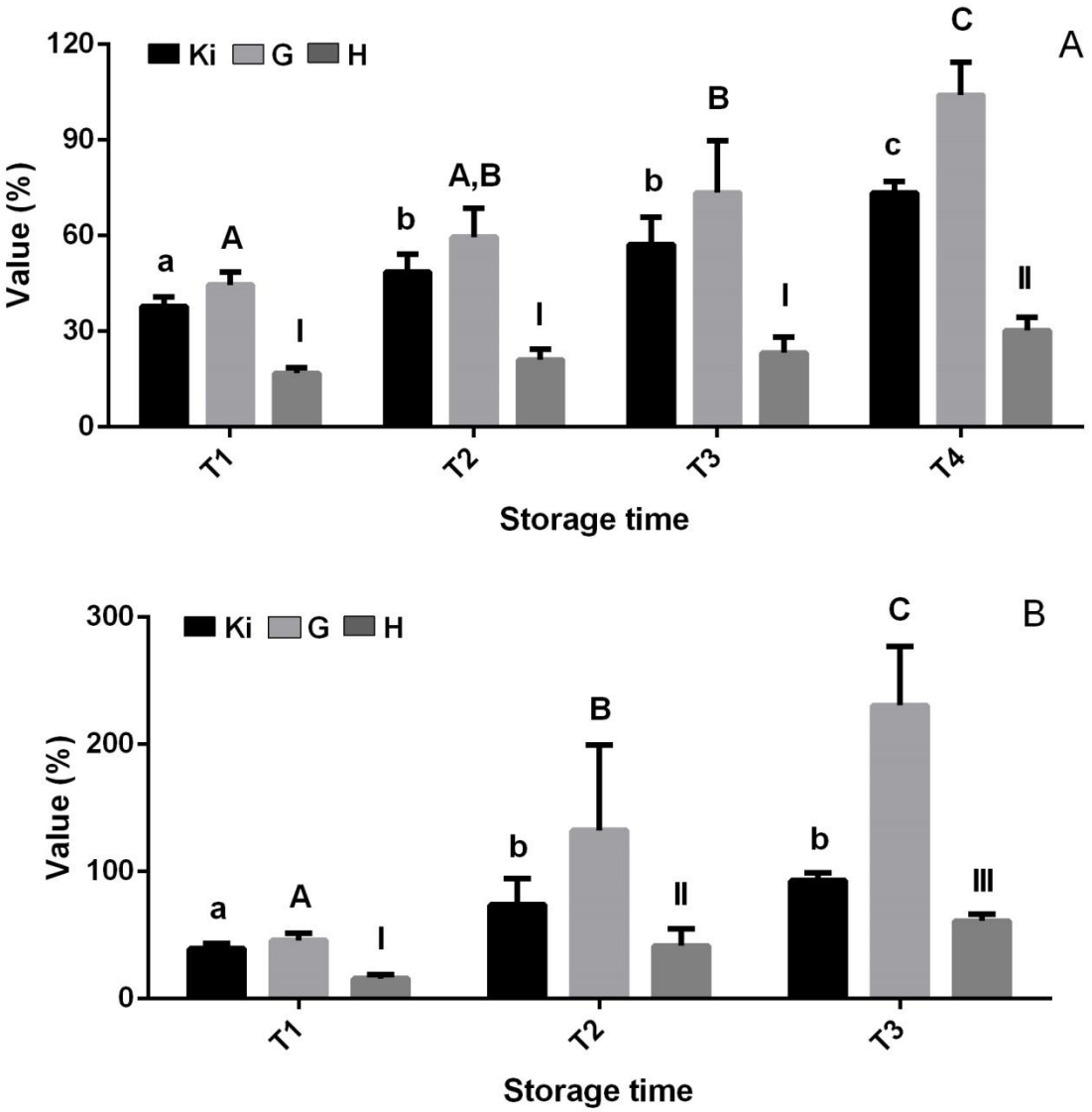

Fig. 4: Ki, G and H values in whitemouth croaker (*Micropogonias furnieri*) kept at 0±1 ºC (A) or 5±1 ºC (B) for different storage times: T1 (0 to 5th storage day), T2 (6th to 10th storage day), T3 (11th to 15th storage day), T4 (16th to 20th storage days). Mean values (n = 4). Standard deviations are indicated by bars. Different lowercase and capital letters and different roman numerals indicate significant differences ($p<0.05$) between values Ki, G and H, respectively.

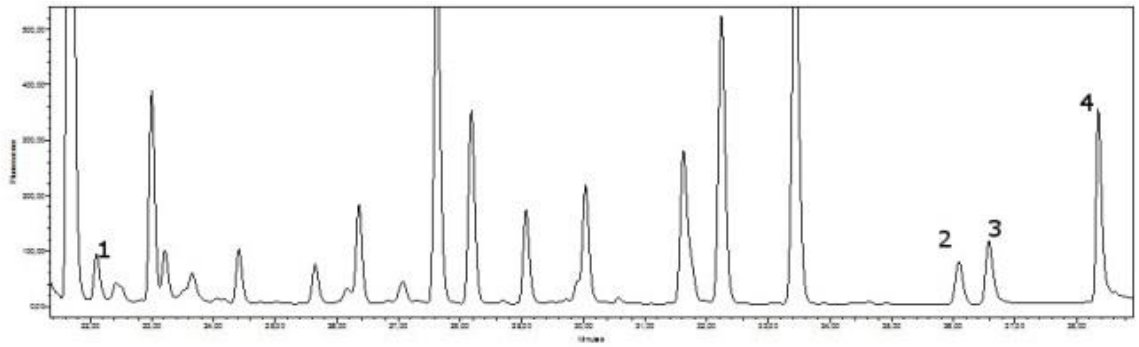

Fig. 5: Typical chromatogram for separation of biogenic amines in samples of refrigerated whitemouth croaker (*Micropogonias furnieri*): 1, histamine; 2, tyramine; 3, putrescine; 4, cadaverine. The other, unidentified peaks are amino acids present in the sample.

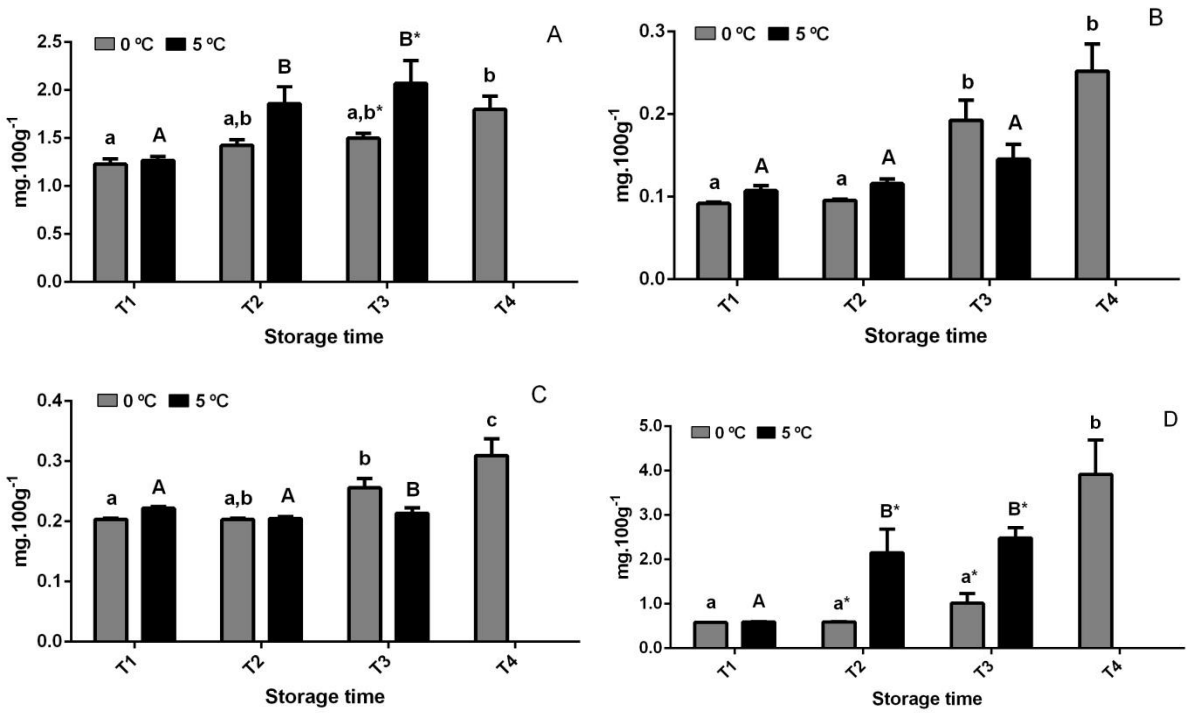

Fig. 6: Levels of the nucleotides histamine (A), putescine (B), cadaverine (C) and tyramine (D) in whitemouth croaker (*Micropogonias furnieri*) kept at 0±1 ºC or 5±1 ºC for different storage times: T1 (0 to 5th storage day), T2 (6th to 10th storage day), T3 (11th to 15th storage day), T4 (16th to 20th storage days). Mean levels (n = 8). Standard deviations are indicated by bars. Different lowercase and capital letters indicate significant differences between values ($p<0.05$) in the samples stored at 0±1 and 5±1 ºC, respectively. * Values with significant differences ($p<0.05$) between the storage temperatures, at the same storage time.

3.3 ARTIGO 3: EFFECT OF STORAGE TEMPERATURE AT THE QUALITY INDEX METHOD SCHEME AND SHELF-LIFE STUDY OF MULLET (*Mugil platanus*). Submitted to Journal of Food Quality.

EFFECT OF STORAGE TEMPERATURE AT THE QUALITY INDEX METHOD SCHEME AND SHELF-LIFE STUDY OF MULLET (*MUGILPLATANUS*)

Quality Index Method and Shelf-life Study of Mullet

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ABSTRACT

A protocol for the sensory assessment of mullet (*Mugilplatanus*) based on the Quality Index Method (QIM) was developed and the influence of storage temperature (0±1 and 5±1ºC) on the Quality Index (QI) and shelf life was verified for 24 and 20 days, respectively. The QI correlated highly with storage time at 0±1ºC (R^2 = 0.982) and 5±1ºC (R^2 = 0.966). In the samples stored at 0±1ºC, all parameters were important, except fin appearance. In mullet stored at 5±1ºC, the parameters gill color (1.256), eye shape (1.198), gill odor (1.139), cornea (1.126), eye area (1.122), odor (1.108) and general appearance (1.073), were the most important. Temperature abuse promoted decrease in eight and 27 fold the lag phase of the mesophiles and psycrotrophs, respectively. According to the sensorial evaluation, the estimated shelf life for mullet stored at 0±1ºC and 5±1ºC was fourteen and seven days, respectively.

Keywords: quality index method, mullet, quality parameters, storage temperature, shelf life.

PRACTICAL APPLICATIONS

The mullet is an important alternative fisheries resource for consumers. However, data regarding the fish quality are scarce. The development of sensorial scheme for mullet based on the Quality Index Method may provide important information on fish quality and its remaining shelf life when stored under different temperatures, which reflect routine fish-handling situations. Therefore, the results of this study can be utilized by researches in future studies as well as by manufacturers to determine the ideal storage conditions of mullet, ensuring thus a reduction in economic losses.

INTRODUCTION

Mullet (*Mugilplatanus*) is a widely distributed pelagic species from the family *Mugilidae* that occurs in tropical and subtropical waters throughout the world, especially in estuaries (Menezes 1983; Miranda and Carneiro 2007). In 2010, this species was among the 11 most caught fish in Brazil, representing more than half (50.7%) of the saltwater fish caught

in the country (Brazil 2012). This species increased the fishery production of the state of Rio de Janeiro by 52.2% in 2007, totaling 1,903.5 tons (IBAMA 2007). After the year 2000, this species became an important alternative to consumers because of the decreasing catch and yield of traditional species (Miranda and Carneiro 2007).

Fish quality is strongly influenced by storage temperature since temperature affects bacterial growth and autolysis (Macagnano *et al.* 2005). Temperature variations due to not covering the fish with ice properly are common and directly impact spoilage rate. Therefore, the capture or storage date alone will not ensure product quality (Taoukis *et al.* 1999; Olafsdottir *et al.* 2006).

The Quality Index Method (QIM) is used for assessing many fish parameters, such as general appearance, texture, eyes, gills, and abdomen, and how these parameters change over time (Sveinsdottir *et al.* 2003; Hyldig and Green-Petersen 2004). The scores given to the different parameters are added, yielding a total demerit score called Quality Index (QI), which varies from one species to another because the QIM is species specific (Huss 1995; Hyldig and Green-Petersen 2004). Specimens are not rejected because of a single parameter, and small changes in the score of one parameter are not enough to influence the total score (Huidobro *et al.* 2000; Sykes *et al.* 2009).

QIMs have been developed for many fish species, such as sardines (*Sardina piuchardis*, *Sardinops sagax*, *Sardinella brasiliensis* and *Cetengraulis edentulus*) (Triqui and Bouchriti 2003; Musgrove *et al.* 2007; Andrade *et al.* 2012), hake (*Merluccius merluccius*) (Baixas-Nogueiras *et al.* 2003), salmon (*Salmo salar*) (Sveinsdottir *et al.* 2003), octopus (*Octopus vulgaris*) (Barbosa and Vaz-Pires 2004), anchovies (*Engraulis encrasicholus*), (Pons-Sánchez-Cascado *et al.* 2006), cod (*Gadus morhua*) (Bonilla *et al.* 2007), cuttlefish (*Sepia officinalis*) (Sykes *et al.* 2009), shrimp (*Litopenaeus vannamei*) (Oliveira *et al.* 2009), croaker (*Micropogonias furnieri*) (Teixeira *et al.* 2009), gilthead sea bream (*Sparus aurata*) (Huidobro *et al.* 2000; Campus *et al.* 2011) and bogue (*Boops boops*, L.) (Bogdanović *et al.* 2011). However, studies that include storage temperature are scarce. Therefore the development of sensorial scheme for mullet ready to use for scientific and industrial freshness assessment may provide important information on fish quality and its remaining shelf life when stored under different temperatures, which reflect routine fish-handling situations.

This study aims to develop a QIM for assessing mullet (*Mugilplatanus*) and determine the effect of storage temperature (0±1 and 5±1ºC) on its quality index and shelf life.

MATERIAL AND METHODS

Fish, storage conditions and experimental design

Mullet weighing between 389.3 and 550.7 grams were purchased from fishing boats at the municipality of Maricá, lake region of the state of Rio de Janeiro, from May to August 2012. The mean ambient water temperature was 23±1ºC. Immediately after capture, the mullet were killed by immersion in ice-water slurry. After death, fish were placed inside expanded polystyrene coolers with ice and transported to the laboratory within two hours where they were rinsed individually. At the laboratory the fish were subdivided in two groups. The first group consisted of 36 fish which were placed in plastic containers with ice (0±1ºC) and stored in a refrigerator (5±1ºC). More ice was added as needed. The second group consisted of 20 fish which were placed in plastic containers and stored in a refrigerator (5±1ºC). Twelve fish stored at 0±1ºC were used for training the sensory panel. In the first training period, the fish were assessed after 2, 6, 9, 13, 16, 20, and 22 days of storage and in the second training period, after 0, 3, 6, 10, 13, 17, 20, and 24 days of storage. Fourteen fish

kept at 0±1ºC after 0, 4, 7, 11, 14, 17, and 21 days of storage were analyzed with QIM; therefore ten fish kept at 5±1ºC were analyzed with QIM after 0, 4, 7, 10, and 14 days of storage. Ten fish stored at 0±1ºC and 5±1ºC for 24 and 20 days, respectively, were used for microbial counts.

Bacterial counts

The bacterial counts were performed every other day in samples separated for this purpose but stored under the same conditions as the other samples. A sample of 25g of each specimen was collected under sterile conditions, homogenized with 225mL of 0.1% saline peptone water by a stomacher (SEWARD – Stomacher 80), and diluted by a factor of 10, from which other dilutions were produced. The aerobic mesophilic heterotrophic and psychrotrophic bacteria (AMHB and AMPB, respectively) were counted as recommended by the American Public Health Association (APHA, 2001) which requires spreading 1mL of the different dilutions on standard plate count agar (PCA). AMHB were incubated at 35-37ºC for 48 hours and AMPB at 4±1ºC for seven days.

Quality index method for mullet

The QIM was developed as suggested by Sveinsdottir *et al.* (2003). A team of six panelists, three males and three females, aged 24 to 40 years was trained under laboratory conditions and asked to develop a QIM protocol. The training sessions lasted about one hour each. The samples were removed from the ice 30 minutes before the training session and placed in white plastic trays. The panelists examined and recorded the changes that occurred in the fish over time until the fish was no longer acceptable for consumption. They then selected by consensus the parameters that should compose the quality index (QI). The score given to each parameter varied from 0 to 3.

At the end of the training period, the mullet samples stored at 0±1^{o}C were placed in light-colored trays numbered with random 3-digit numbers and presented to the panelists under standardized laboratory conditions. Each panelist analyzed the sample individually and recorded the score given to each parameter in the QI. The same procedure was followed for the mullet samples stored at 5±1^{o}C.

Statistical analysis

The bacterial growth variables ‘lag phase’ and ‘generation time’ were analyzed by the software DMfit 2.0 based on predictive microbiology and idealized by Baranyi and Roberts (1994).

The correlation between the sensory changes and storage time was determined by linear regression of the QIM data. One-way analysis of variance (one-way ANOVA) and the Tukey test determined the differences between the parameters of the samples stored under different temperatures and storage times. Partial least squares (PLS) regression determined the shelf life of the species and the most important sensory parameters for the QIM. All analyses were done by the software XLSTAT 2013.1 (Adinsoft, Paris, France), with a significance level of 5%.

RESULTS AND DISCUSSION

Bacterial counts

Figure 1 shows that AMHB and AMPB counts increased exponentially over storage time, regardless of temperature, but more steeply in samples stored at 5±1°C.
On storage day 0 (capture date), the mean AMHB was 2.2 log CFU.g^{-1} and AMPB was 1.9 log CFU.g^{-1}. The acceptable upper limit count of 7.0 log CFU.g^{-1} established by the International Commission of Microbiological Specifications for Foods (ICMSF, 1986) was reached after 20 days in samples stored at 0±1°C and after 14 days in samples stored at 5±1°C. Other studies (Erkan and Özden 2008; Andrade 2012, Chong *et al.* 2012) obtained different results, which may be justified by the lower baseline bacterial load of the fish used in the present study.

Table 1 shows that temperature abuse promoted a decrease in the lag phase and generation time of the study bacteria. The lag phases of the AMHB and AMPB in samples stored at 0±1°C were 7.5 and 2.7 days, respectively, which were eight and a twenty-seven times higher than those of samples stored at 5±1°C (0.9 and 0.0, respectively), increasing the shelf life of mullet stored at 0±1°C. Additionally, the greater amount of time required by mesophiles to adapt indicates that refrigeration facilitated the development of psychrotrophic bacteria, regardless of temperature. At 5±1°C, these bacteria did not require an adaptation phase to grow, probably because the conditions were favorable.

The present and other studies found that higher storage temperatures facilitate the growth of microorganisms in fish and consequently reduce their shelf life (Gram and Huss 1996; Veciana-Nógues *et al.* 1997; Taoukis *et al.* 1999; Millán *et al.* 2003; Olafsdottir *et al.* 2006; Boziaris *et al.* 2011). The generation times of mesophiles (20.1 hours) and psychrotrophs (11.3 hours) were lower in samples stored at 5±1°C than in those stored at 0±1°C (22.6 and 13.8 hours, respectively). Ababouch *et al.* (1996) also studied the influence of storage temperature on the generation time of bacteria in sardines (*Sardina pilchardus*). In samples stored at room temperature, the generation times of mesophiles (1.9 hours) and psychrotrophs (3.2 hours) were smaller than those of samples stored in ice (16.0 and 14.5 hours, respectively).

Quality index

The quality index (QI) protocol for the sensory assessment of mullet (*Mugilplatanus*) (Table 2) stored at 0±1°C contains the parameters suggested by a team of six trained panelists. Thirteen parameters were selected, namely general appearance, scale adherence, flesh firmness, odor, cornea, pupil, eye shape, eye area, gill color and odor, abdomen, and fin appearance and flexibility. The demerit scores ranged from 0 to 1, 0 to 2, and 0 to 3, depending on parameter, 0 being the best and 3 being the worst. The total score for mullet was 33 demerit points. The QI was calculated for each storage day by adding the scores given by the panelists. The inclusion of parameters specific to the study species, such as eye area and fin appearance and flexibility resulted in a higher total QI demerit score than that for other species (Sveinsdottir *et al.* 2002; Bonilla *et al.* 2007; Teixeira *et al.* 2009; Andrade *et al.* 2012; Bogdanović *et al.* 2012). Although Massa *et al.* (2005) and Sant'Ana *et al.* (2011) included different parameters, they suggested 30 and 32 demerit points for the sensory assessment of red sea bream (*Pagellus bogaraveo*) and Patagonian flounder (*Paralichthys patagonicus*), respectively.

The mean scores of the mullet stored at 0±1°C on days 0 and 21 were 0.50±0.55 and 19.33±1.63, respectively. On the other hand, the mean scores of the mullet stored at 5±1°C on days 0 and 14, the last assessment day, were 2.00±1.90 and 21.50±0.55, respectively. Regardless of storage temperature, the sensory characteristics of mullet changed more slowly during the intermediate storage period, that is, between days seven and ten for mullet stored at 0±1°C and between days four and seven for mullet stored at 5±1°C, confirmed by the similar mean scores obtained on those days. The QI correlated highly with storage time for samples stored at 0±1°C (R^2 = 0.982) and 5±1°C (R^2 = 0.966) (Figure 2).

The sensory characteristics of mullet changed faster in samples stored at 5±1°C than in those stored at 0±1°C. Higher storage temperature facilitates bacterial growth and biochemical reactions with consequent sensory changes, as reported by many authors (Ababouch *et al.*; Millán *et al.* 2003; Guizani *et al.* 2005; Olafsdottir *et al.* 2006; Boziaris *et al.* 2011). The scores given by the panelists show that very fresh mullet has a QI score of 0 to 4; scores higher than 16 suggest that the fish is no longer fit for human consumption. Such scores occur after fourteen and seven days of storage at 0±1°C and 5±1°C, respectively.

Figure 3 shows that the demerit scores of all parameters increased as a function of storage time, regardless of temperature. However, general appearance, flesh firmness, odor, cornea, eye area, gill odor, and gill color changed significantly faster in samples stored at 5±1°C. The smell of seawater on mullet gills and scales appeared on the fourth day of storage at 5±1°C, while the samples stored at 0±1°C still smelled like sea algae. On the tenth storage day, the flesh of the mullet stored at 5±1°C was soft and the cornea opaque, while those of the mullet stored at 0±1°C were firm and slightly opaque, respectively. The mullet stored at 5±1°C were rejected sensory on storage day 14. They had lost their metallic gloss, their eye area was reddish, and the gills brownish. On the fourteenth day of storage, the mullet stored at 0±1°C still had some of their metallic gloss, a yellowish eye area, and brownish red gills; they were not rejected.

Partial Least Squares (PLS) regression established the correlations between mullet stored at 0±1°C and 5±1°C. The standard error associated with the prediction of the storage time was then calculated and the important QIM parameters identified. The two models generated by PLS regression presented appropriate QI values, which ranged from 0.899 (0±1ºC) to 0.920 (5±1ºC), explaining 90.6% and 95.1% of the data variability as storage temperature increased. Only one dimension was necessary for generating a PLS model for the 0±1°C data, while two dimensions were used for the 5±1°C data, which may be related to the variations associated with higher storage temperatures, which impair the panelists' assessments.

The standard error associated with the prediction of the storage time of the samples stored at 0±1°C and 5±1°C was of two (2.15) and one (0.94) days, and the coefficients of determination (R^2) were 0.906 and 0.967, respectively. Similar findings were observed for pacu fish (*Piaractus mesopotamicus*) (Borges *et al.* 2013). The different predicted storage times of mullet stored under different temperatures may be related to variations in the scores given by the panelists to each parameter, especially for samples stored at 0±1°C (Figure 4A). This score variation did not occur for the samples stored at 5±1°C (Figure 4B), meaning that the scores given by the panelists to these samples were more homogeneous during the entire storage period. This can be attributed, according Sveinsdottir *et al.* (2003) and Borges *et al.* (2013), to speed of sensory changes that occurred more rapidly in samples stored at 5±1ºC when compared to samples stored at 0±1ºC, and therefore more easily perceptible.

A Variable Importance in the Projection (VIP) greater than 1.0 revealed which parameters were important for the model (Donadoni *et al.* 2012). The VIP indices are also of paramount interest as they highlight the importance of the various attributes. They may be

useful in guiding the selection of a subset of relevant sensory descriptors from the complete set of attributes; VIPs are associated with attributes, and reflect their contribution in discriminating the products (Rossini *et al.* 2013). Except for fin appearance, all parameters of the samples stored at 0±1°C were important for mullet's QIM, especially gill odor (1.210), odor (1.151), cornea (1.145), eye shape (1.083), general appearance (1.067), fin flexibility (1.049), and gill color (1.001). For the samples stored at 5±1°C, the parameters scale adherence, flesh firmness, and abdomen were not important for mullet's QIM. However, the parameters gill color (1.256), eye shape (1.198), gill odor (1.139), cornea (1.126), eye area (1.122), odor (1.108), and general appearance (1.073) were the most important.

Figures 5A and 5B show the parameter importance for the QIM of mullet stored at 0±1°C and 5±1°C, respectively, according column height (Cadena *et al.* 2012). Storage temperature determined which parameters were important and their importance in the QIM. In mullet stored at 0±1°C, all parameters were important, but the parameters gill odor, odor, cornea, eye shape, general aspect, fin flexibility, and gill color contributed most. On the other hand, in mullet stored at 5±1°C, the parameters gill color, eye shape, gill odor, cornea, eye area, odor, and general appearance were important, while the parameters scale adherence, abdomen, and fin appearance and flexibility were not important.

These results may be useful for all segments involved in mullet handling and storage because, clearly, visual parameters are easy to assess and effectively reflect the degree of freshness of mullet, enabling the selection of similar quality products for processing. It also makes assessment easier, since trained panelists can focus exclusively on important parameters.

Shelf life

The fish shelf life can be determined by physical-chemical and microbiological analysis, however sensory changes that occur in this product during the storage period has been commonly used for this purpose because infer about your limit of acceptability (Massa *et al.* 2012; Borges *et al.* 2013; Giuffrida *et al.* 2013). In the present study, given the capture location and conditions, the low baseline microbial load, and appropriate storage temperature, the results suggest that chemical reactions played a greater role in spoilage than microbial growth.

AMHB reached the highest acceptable limit recommended by the International Commission of Microbiological Specifications for Foods (ICMSF, 1986) (7 log $CFU.g^{-1}$) on storage days 20 and 14 for the samples stored at 0±1°C and 5±1°C, respectively, but the samples had already been rejected on days 17 and 10, respectively. Similar results were found for tuna (*Thunnus albacores*) (Guizani *et al.* 2005) and bream (*Megalobrama amblycephala*) (Song *et al.* 2012). According to these authors, rejection depended not only on bacterial count, but also on the type of bacteria present, degree of autolysis, intrinsic quality parameters, and storage conditions. Each fish species has its own spoilage bacteria, and it is their number that determines shelf life, not the total bacterial load (Huss 1995).

Hence, sensory assessment and AMHB count will establish the shelf life of mullet stored at 0±1°C and 5±1°C. However, 7 log $CFU.g^{-1}$ should not be considered the maximum acceptable bacterial load for this species because the samples were rejected before this limit was reached. The fish were rejected when their mean QIM score using the parameters proposed by this study reached 16. At this point, the bacterial load was 4 log $CFU.g^{-1}$. Therefore, the shelf life of fourteen and seven days for mullet stored at 0±1°C and 5±1°C, respectively.

CONCLUSION

Temperature abuse promoted decrease of lag phase in eight and 27 fold the lag phase of the mesophiles and psycrotrophs, respectively.

The QIM developed for mullet stored under different temperatures enabled appropriate assessment of the sensory parameters of this species and can be used in different mullet handling stages. In particular, the sensory attributes gill color, eye shape, gill odor, cornea, eye area and superficial odor showed relevant due to change on temperature storage.

PLS regression may be used by the scientific and productive sectors, as it correctly predicts the shelf life of mullet stored under different temperatures and indicates which parameters are important at a given condition, reducing the time and cost required to train a team of panelists. The present results are also useful for the fish industry. Future studies should perform the sensory profiling using quantitative descriptive analysis (QDA) of cooked mullet to give better understanding of the situation.

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TABLE 1: AEROBIC MESOPHILIC HETEROTROPHIC (AMHB) AND PSYCHROTROPHIC (AMPB) BACTERIAL COUNTS IN MULLET (*Mugilplatanus*) STORED AT 0±1 AND 5±1ºC FOR 24 AND 20 DAYS, RESPECTIVELY.

	Storage temperature	Baseline count (log $CFU.g^{-1}$)	Lag (days)	Generation time (hours)	BBC at the BSP (log $CFU.g^{-1}$)
CBHM	0±1ºC	2.2	7.6	22.6	7.2
	5±1ºC	2.2	0.9	20.1	8.4
CBHP	0±1ºC	1.9	2.7	13.8	11.5
	5±1ºC	1.9	0.0	11.3	12.7

BBC at the BSP: baseline bacterial count at the beginning of the stationary phase.

TABLE 2: QUALITY INDEX (QI) PROTOCOL FOR ASSESSING MULLET (*Mugilplatanus*) STORED AT 0±1ºC AND 5±0ºC.

Parameters		Characteristics	Score
General appearance	General appearance	Intense metallic gloss, distinct colors (teal in the dorsal area, yellowish stripes on the sides, pearl on the abdomen)	0
		Not so intense metallic gloss, loss of the yellowish color, pinkish abdomen	1
		Loss of metallic gloss, blood stains on the head	2
	Scales	Well attached	0
		Loose	1
	Flesh firmness	Firm	0
		Slightly soft	1
	Odor	Saltwater algae	0
		Seawater	1
		Rancid	2
		Putrid	3
Eyes	Cornea	Transparent	0
		Slightly opaque	1
		Opaque	2
	Pupil	Oval with sharp borders	0
		Loss of border sharpness	1
	Shape	Convex	0
		Flat	1
		Concave	2
	Eye area (external)	Clear	0
		Opaque, yellowish	1
		Reddish	2
Gills	Odor	Saltwater algae	0
		Sea water	1
		Rancid	2
		Putrid	3
	Color	Bright red	0
		Brownish red	1
		Brownish	2
Abdomen	Appearance	Firm	0
		Soft	1
Fins	Appearance	Intact	0
		Injured	1
	Flexibility	Flexible	0
		Inflexible	1
Total score			0-33

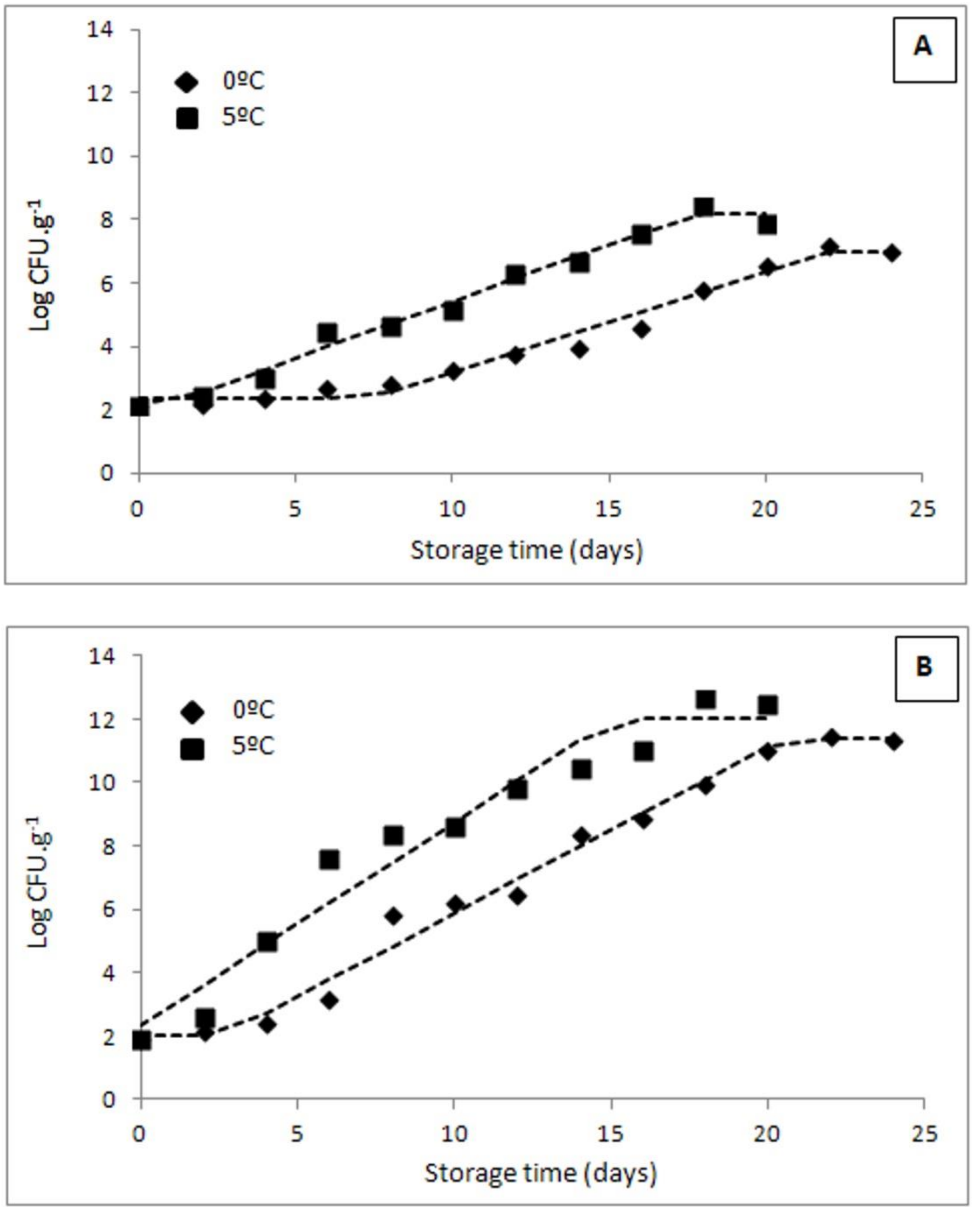

Figure 1: AEROBIC MESOPHILIC HETEROTROPHIC (AMHB) (A) AND PSYCHROTROPHIC (AMPB) (B) BACTERIAL COUNTS IN MULLET (*Mugil platanus*) STORED AT 0±1 AND 5±1ºC FOR 24 AND 20 DAYS, RESPECTIVELY.

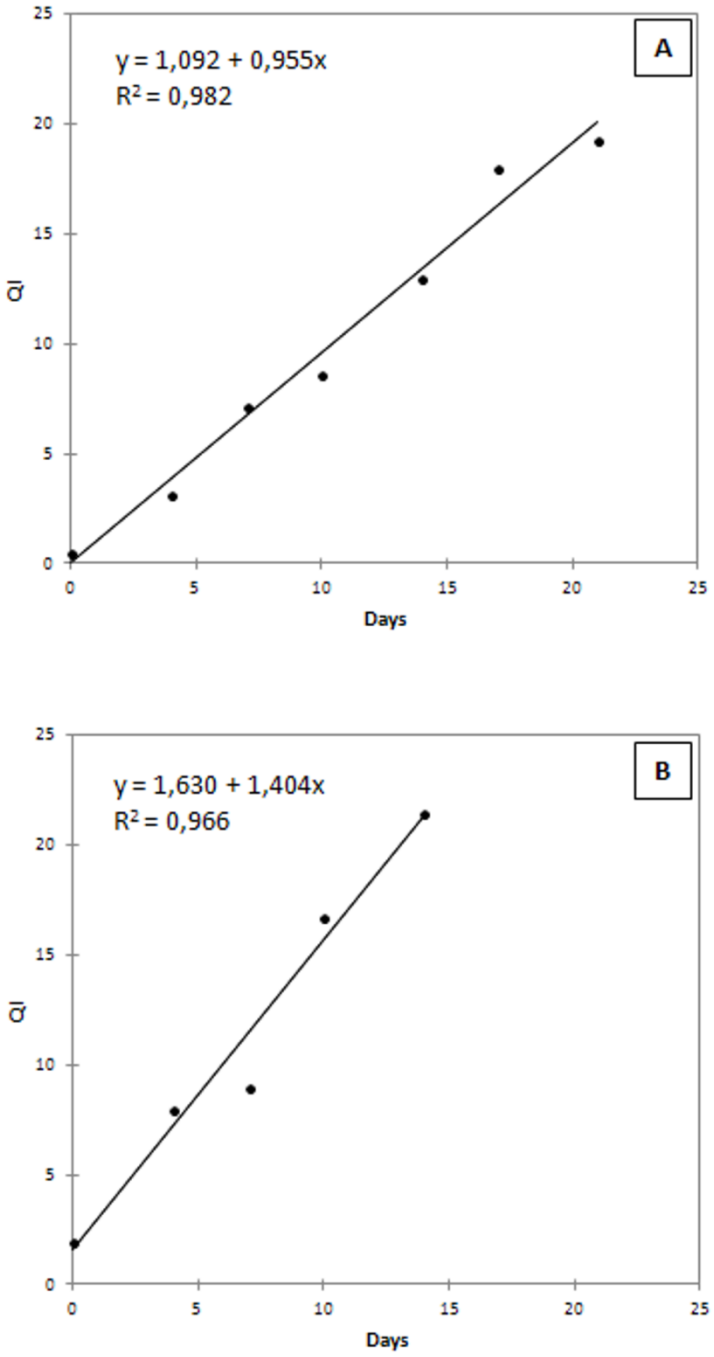

Figure 2: LINEAR CORRELATION BETWEEN MEAN QI AND STORAGE TIME OF MULLET (*Mugil platanus*) STORED AT 0±1ºC (A) AND 5±1ºC (B).

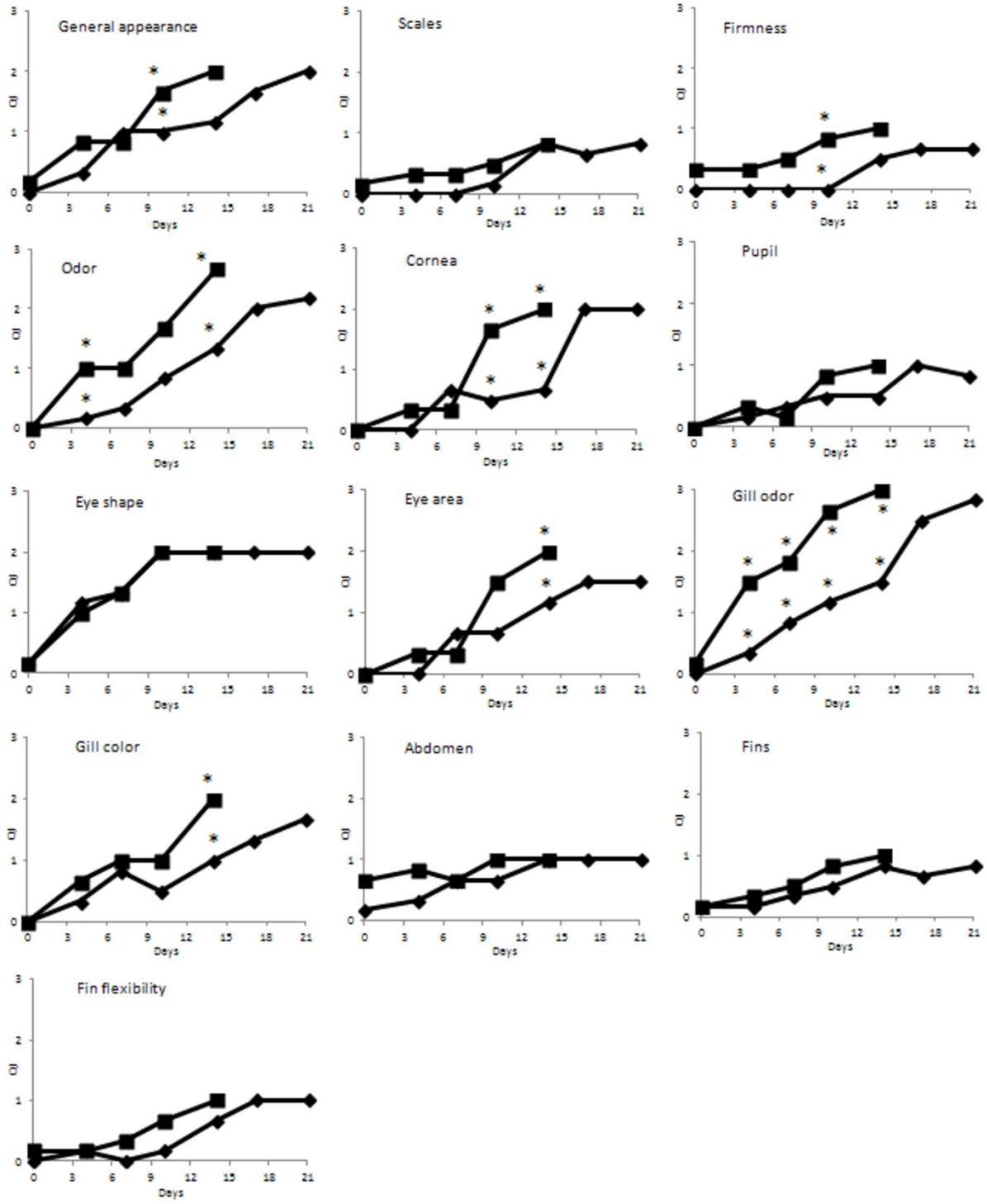

Figure 3: MEAN SCORE OF EACH PARAMETER AS A FUNCTION OF THE STORAGE TIME OF MULLET (*Mugil platanus*) STORED AT 0±1ºC (●) AND 5±1ºC (■) (* PARAMETERS THAT PRESENTED A SIGNIFICANT DIFFERENCE BETWEEN TEMPERATURE AT THE 95% SIGNIFICANCE LEVEL).

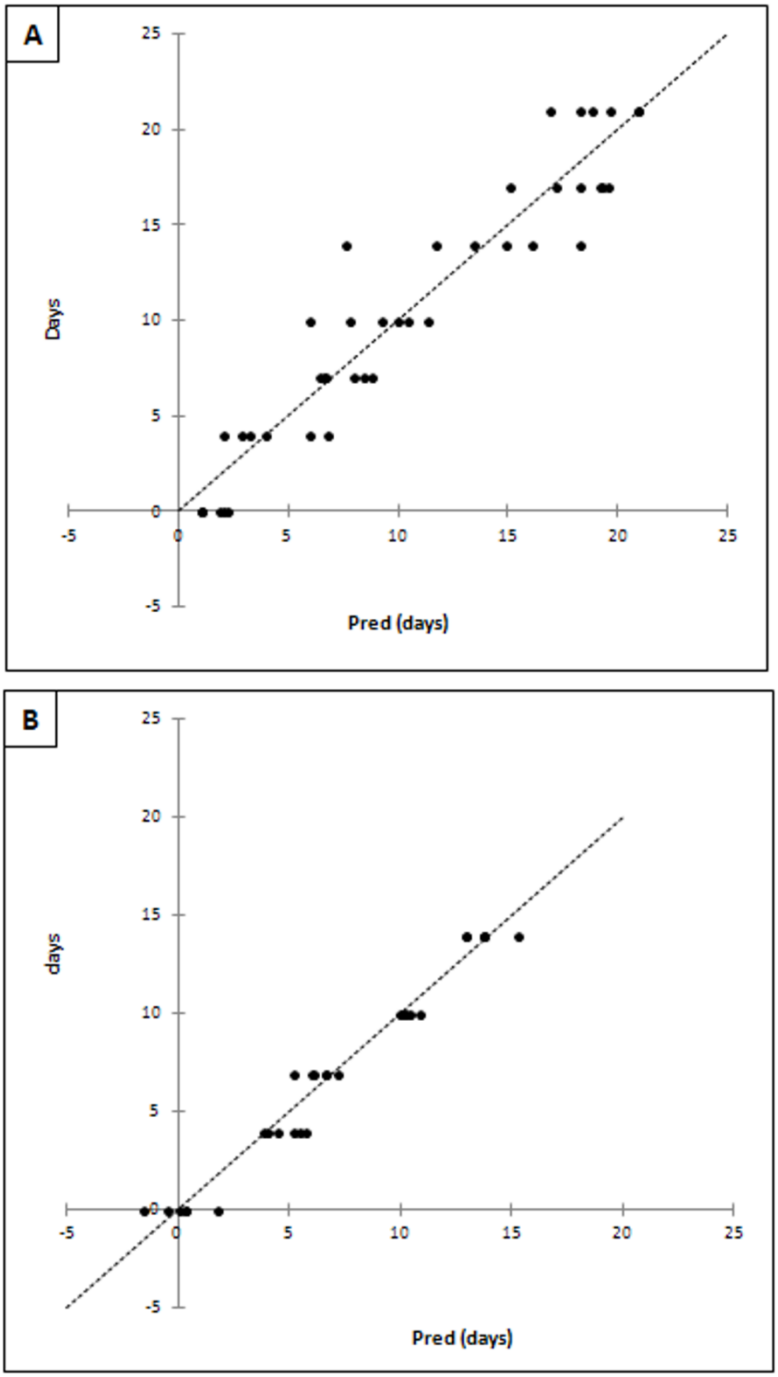

Figure 4: PARTIAL LEAST SQUARES (PLS) REGRESSION OF THE QUALITY INDEX METHOD (QIM) FOR MULLET (*Mugil platanus*) STORED AT 0±1ºC (A) AND 5±1ºC (B).

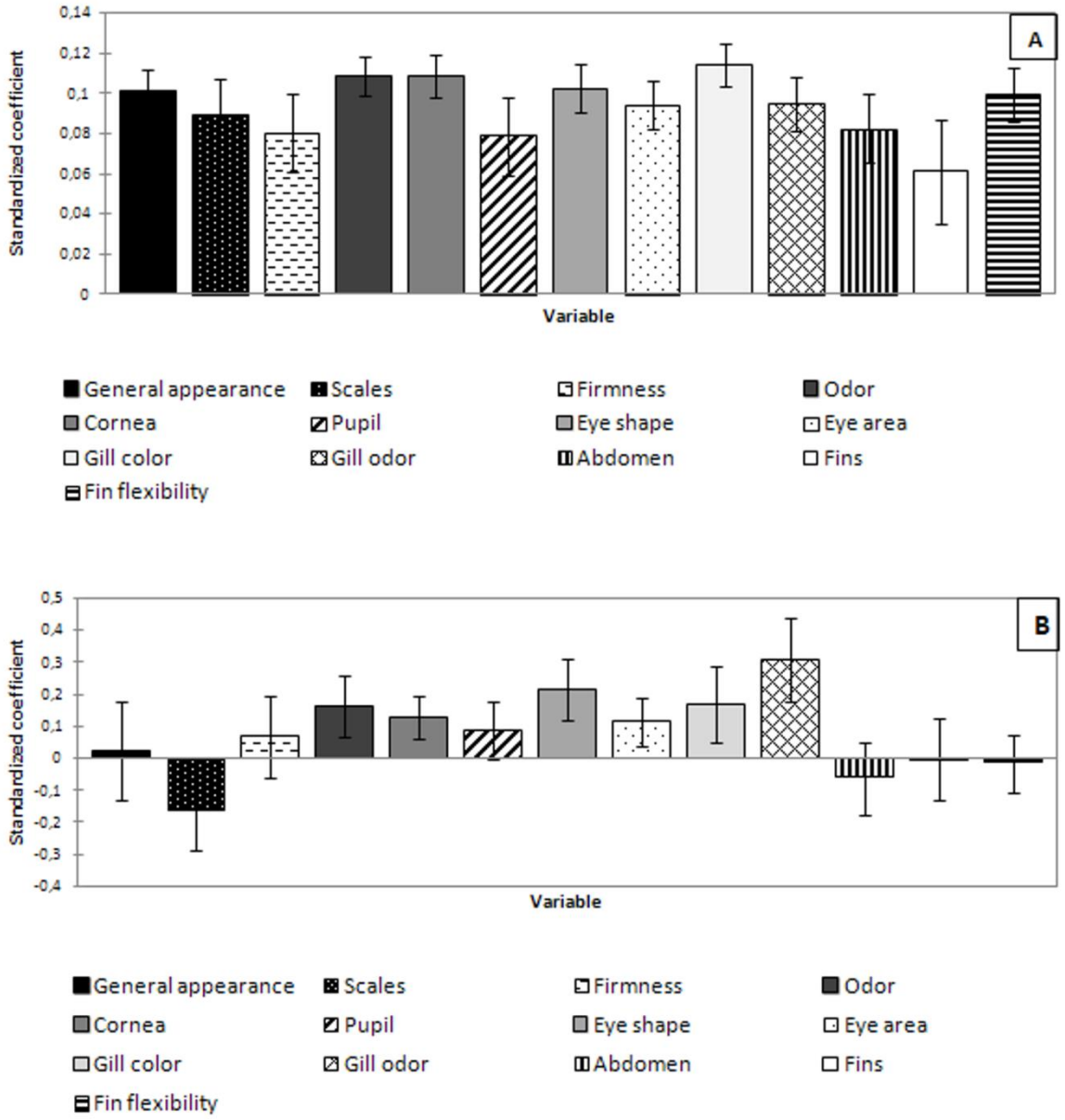

Figure 5 – COEFFICIENT OF THE PARTIAL LEAST SQUARES (PLS) REGRESSION OF THE PARAMETERS IN THE QUALITY INDEX METHOD (QIM) FOR MULLET (*Mugilplatanus*) STORED AT 0±1ºC (A) AND 5±1ºC (B).

4 CONSIDERAÇÕES FINAIS

Pesquisas relacionadas à qualidade do pescado e derivados ainda merecem destaque, tendo em vista que estas espécies apresentam predisposição à deterioração em função de condições apropriadas para ocorrência de alterações degradativas nas moléculas que o compõe. Ao perder a integridade molecular e, consequentemente, a qualidade, são originados metabólitos que caracterizam deterioração e risco àsaúde humana.

Dentre os parâmetros disponíveis para avaliar a qualidade, a avaliação sensorial constitui uma importante ferramenta auxiliando na determinação da validade comercial. A partir do protocolo sensorial elaborado no método de índice de qualidade a avaliação da tainha foi direcionada aos parâmetros de qualidade que se alteram no decorrer do período de estocagem. Desta forma, esse protocolodemonstrou ser adequado para ser aplicado pelas indústrias, pois permite a minimização dos gastos com treinamento de equipe e com análises instrumentais, que poderão ser realizadas apenas noscasos de dúvidas nos resultados.

O índice de qualidade em torno de 16 foi estabelecido como limite de aceitabilidade sensorial da tainha. Desta forma, a validade comercial desta matriz alimentar de acordo com a rejeição sensorial dos julgadores ocorreu num período de tempo inferior ao alcance do limite da contagem de bactérias mesófilas estabelecida na legislação internacional. A avaliação sensorial é um método rápido e prático, no entanto subjetivo, pois depende das diferentes percepções dos avaliadores. Desta forma, a avaliação sensorial auxiliará na determinação da validade comercial do produto, porém a rejeição do produto deve ser feita com base nas análises físico-químicas e microbiológicas, que são mais precisas.

Pequenas variações na temperatura de estocagem foram capazes de diminuir em vários dias a validade comercial dos peixes analisados. O aumento da temperatura de armazenamento ocasionou o aumento da velocidade das reações bioquímicas e degradação microbiológica da matriz alimentar, possivelmente em função da atuação mais rápida das enzimas nos processos degradativos.

Considerações relacionadas a procedimentos analíticos objeto deste estudo, incluem a degradação dos nucleotídeos nas duas espécies de peixe analisadas a qual apresentou adequada correlação com o tempo de estocagem, sendo este

processo acelerado com o aumento da temperatura. No entanto, observou-se que o padrão de degradação destes compostos variou de acordo com a espécie avaliada. O IMP e a Hx foram considerados adequados para serem utilizados como parâmetros químicos na avaliação da qualidade tanto da corvina como da tainha. Entretanto, a HxR demonstrou ser um importante parâmetro indicativo de perda da qualidade da tainha. Desta forma, concentrações de IMP e Hx abaixo e acima de 4.0 $\mu mol.g^{-1}$, respectivamente, são sugestivos de perda da qualidade da corvina. Na tainha, a ausência de IMP indica perda de frescor desta espécie, entretanto o limite de aceitabilidade de consumo para esta matriz alimentar pode ser estabelecido baseado em concentrações de Hx acima de 3.0 $\mu mol.g^{-1}$.

Os índices de qualidade (Ki, G e H) apresentam elevada correlação com o tempo de estocagem em ambas as espécies, aumentando no decorrer do período de armazenamento. Sugere-se que a corvina muito fresca apresente valores Ki < 40%, G<45% e H< 18%; ainda aptas para consumo, aquelas com valores 40% < Ki < 60%, 45% < G < 75% e 18% < H <32%; e em início de putrefação, corvinas com valores Ki > 60%, G > 75% e H > 32%. Para a tainha, valores H < 10% foram sugestivos de peixe muito fresco; exemplares com valores 10% < H <50% podem ser considerados ainda aptos para consumo; e valores H > 50% são indicativos de início de putrefação. Os valores Ki e G mantiveram-se estatisticamente constantes após o 10º dia de estocagem, não sendo possível desta forma, sugerir limites de aceitabilidade.

Também se inclui nas análises físico-químicas, a formação das aminas biogênicas que variou em função da espécie avaliada, bem como do tempo e temperatura de estocagem. Na corvina o comportamento e os níveis de histamina e tiramina foram considerados potenciais indicadores de qualidade para avaliação do frescor desta espécie. Sugere-se que níveis acima de 1.50 $mg.100g^{-1}$e 0.7 $mg.100g^{-1}$ para histamina e tiramina, respectivamente, são indicativos de perda de qualidade. Para a tainha o aumento dos níveis de putrescina e tiramina foram indicativos de perda da qualidade. Sugere-se que, para essa espécie, níveis acima de 3.0 $mg.100g^{-1}$e 8.0 $mg.100g^{-1}$ para putrescina e tiramina, respectivamente, sejam considerados como limites de aceitabilidade. A formação das aminas biogênicas na matriz alimentar depende da presença do aminoácido precursor, desta forma,

sugere-se que em estudos futuros, a análise destes compostos seja conduzida de forma que direcione a pesquisa e identificação das aminas biogênicas.

A técnica de cromatografia líquida de alta eficiência utilizada para determinação dos nucleotídeos e quantificação de aminas biogênicas foi adequada, pois permitiu a separação e identificação dos compostos. A utilização da coluna com partículas com partículas de 2,4 µm e a adição do solvente orgânico acetonitrila à fase móvel na análise dos nucleotídeos permitiram a diminuição no tempo de retenção das substâncias avaliadas, com consequente diminuição do tempo e custo de análise e menor quantidade de resíduo da fase móvel descartado. Na análise de aminas biogênicas, o uso do reagente derivatizante AQC permitiu a identificação adequada das aminas biogênicas, no entanto, como este composto também reage com aminoácidos e, sendo a matriz alimentar analisada rica em aminoácidos, os cromatogramas resultantes da análise foram complexos. Apesar de não ter havido interferência na leitura, sugere-se que, em estudos futuros, proceda-se um tratamento prévio da amostra como a extração em fase sólida. Isto também se aplica para a análise de nucleotídeos, uma vez que evitaria uma sobrecarga da coluna analítica.

Por fim, no contexto do presente estudo e considerando-seo crescimento da população mundial, o aumento do consumo de peixes na dieta de todas as parcelas da população e os riscos à saúde inerentes à condição fisiológica, pretende-se sugerir ampliação da linha de pesquisa que deu origem a este estudo, buscando oferecer subsídios aos órgãos oficiais no que diz respeito à readequação dos parâmetros para avaliação da qualidade do peixe, assim como soluções para o Controle de Qualidade ser efetivo e viável quanto àimplementação de tais procedimentos analíticos garantindo a qualidade nutricional, sensorial, bacteriológica e bioquímica do pescado como alimento.

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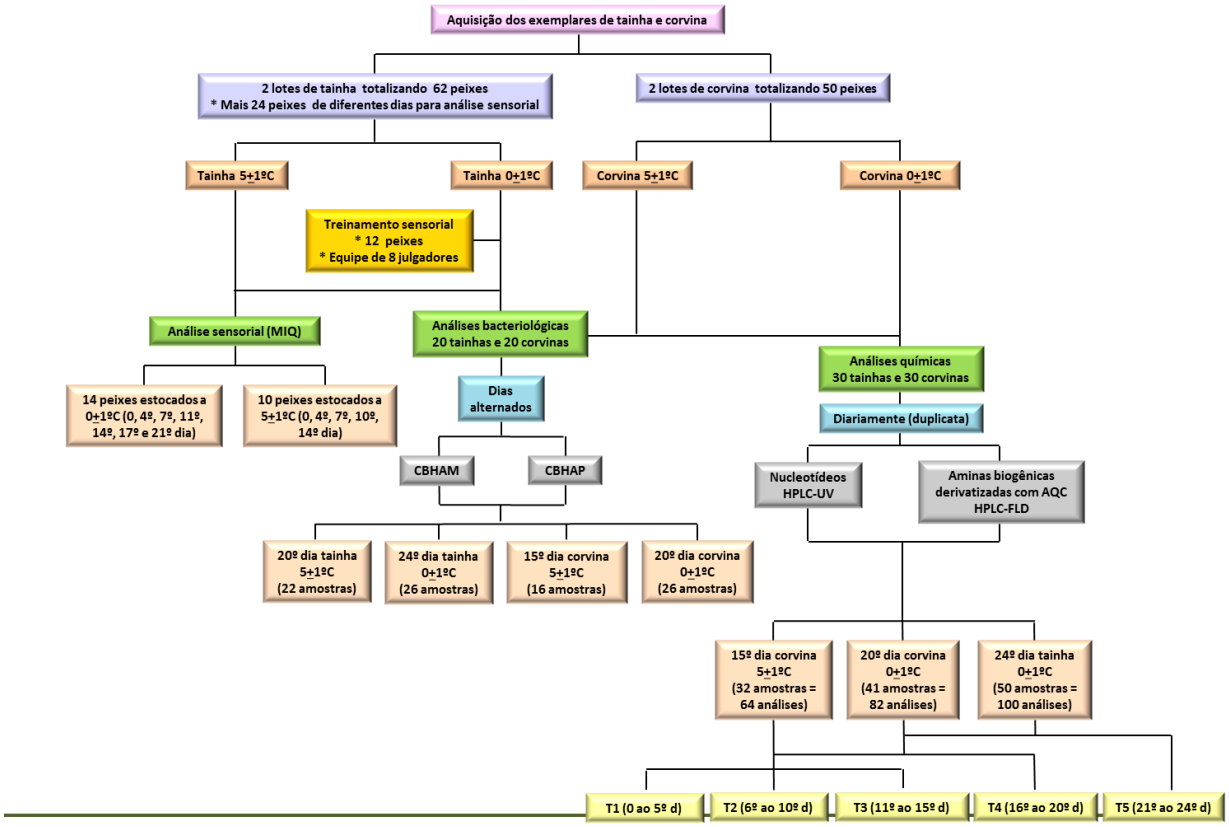
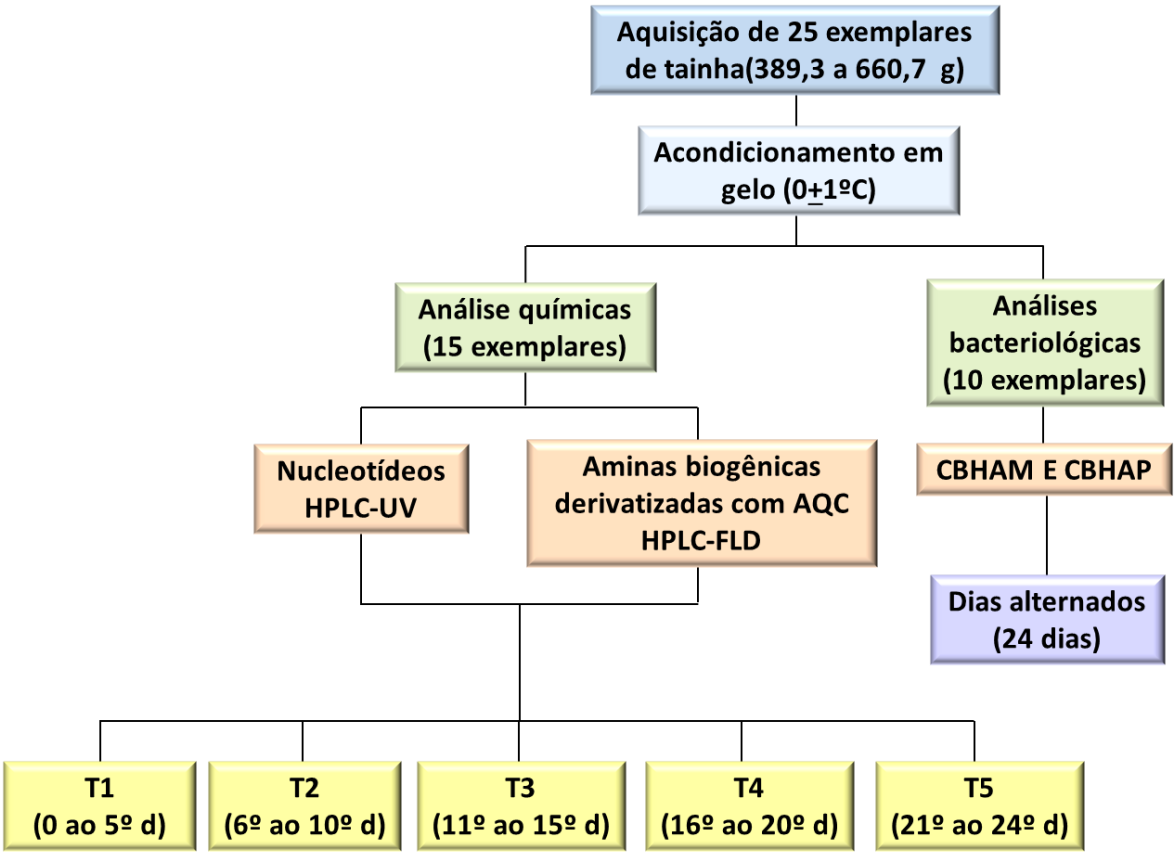
6 APÊNDICE

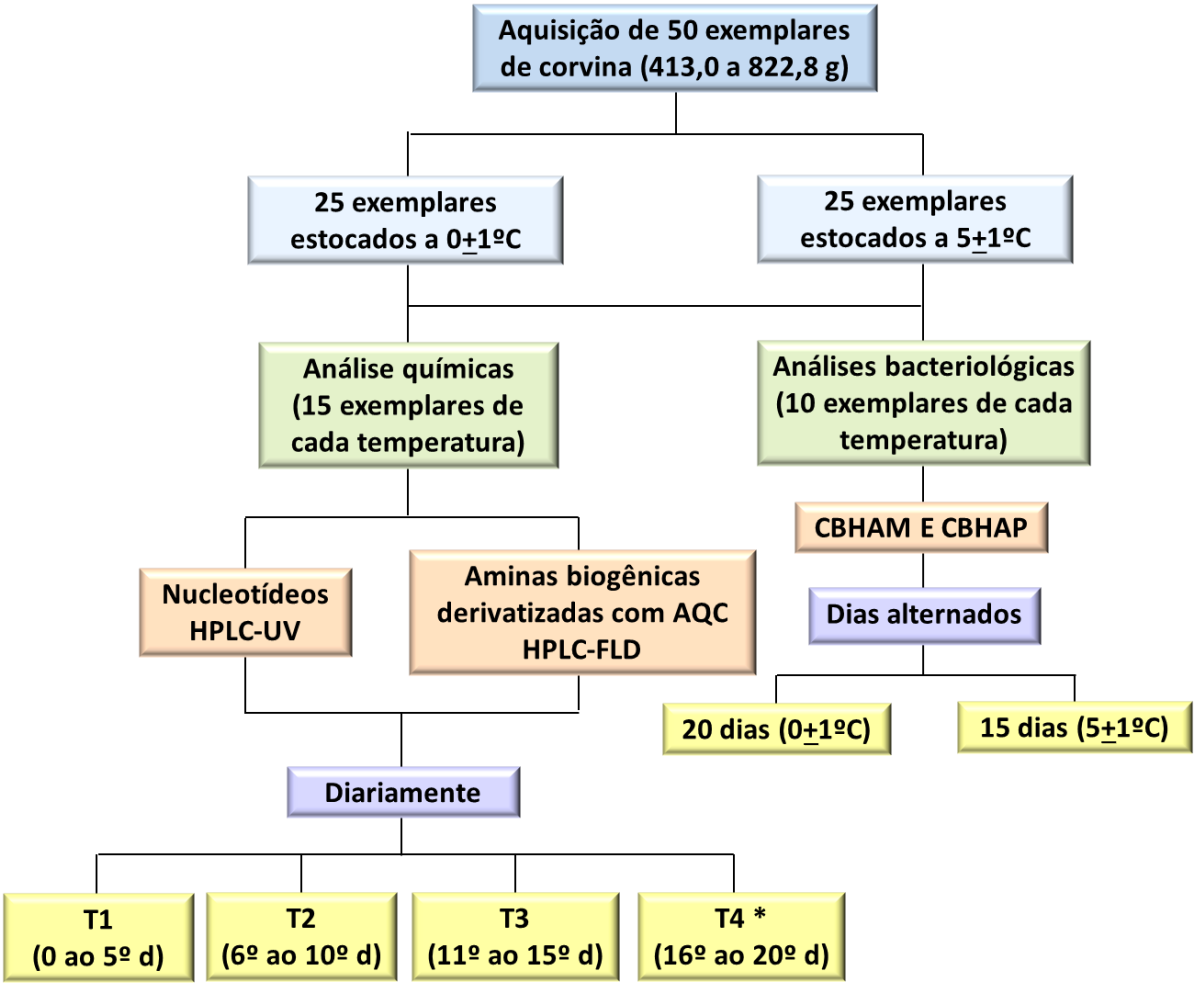
6.1 DESENHO EXPERIMENTAL DELINEADO PARA O DESENVOLVIMENTO DA PESQUISA



6.2 DESENHO EXPERIMENTAL DELINEADO PARA O DESENVOLVIMENTO DO ARTIGO 1

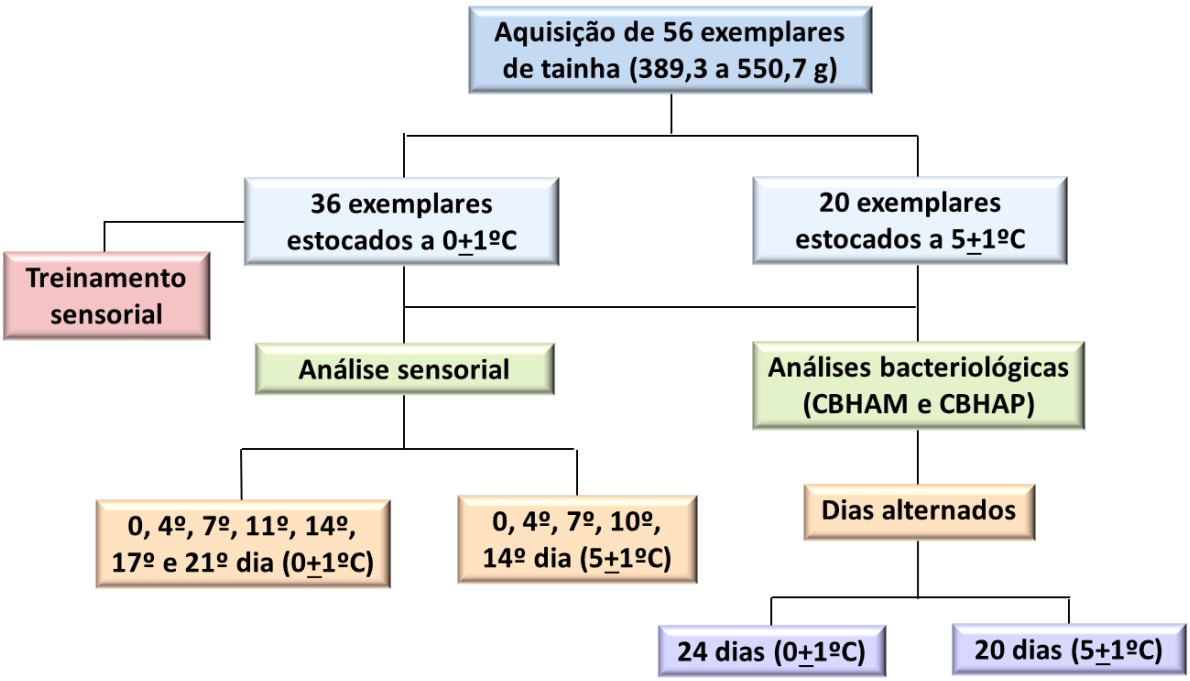


6.3 DESENHO EXPERIMENTAL DELINEADO PARA O DESENVOLVIMENTO DO ARTIGO 2

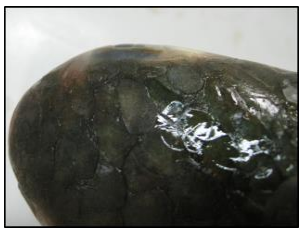
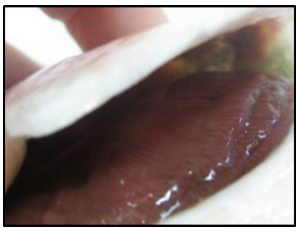


* Amostras estocadas a 0±1ºC

6.4 DESENHO EXPERIMENTAL DELINEADO PARA O DESENVOLVIMENTO DO ARTIGO 3



6.5 PONTUAÇÃO DAS ALTERAÇÕES DOS PARÂMETROS SENSORIAIS DE QUALIDADE DA TAINHA DURANTE PERÍODO DE ESTOCAGEM A 0±1ºC E 5±1ºC

Parâmetro de qualidade	Pontuações		
	0	1	2
Área dos olhos			
Forma dos olhos			
Cor das brânquias			

Fonte: Arquivo pessoal

6.6 PARÂMETROS SENSORIAIS DE QUALIDADE DA TAINHA NO 14º DIA DE ESTOCAGEM A 0±1ºC E 5±1ºC

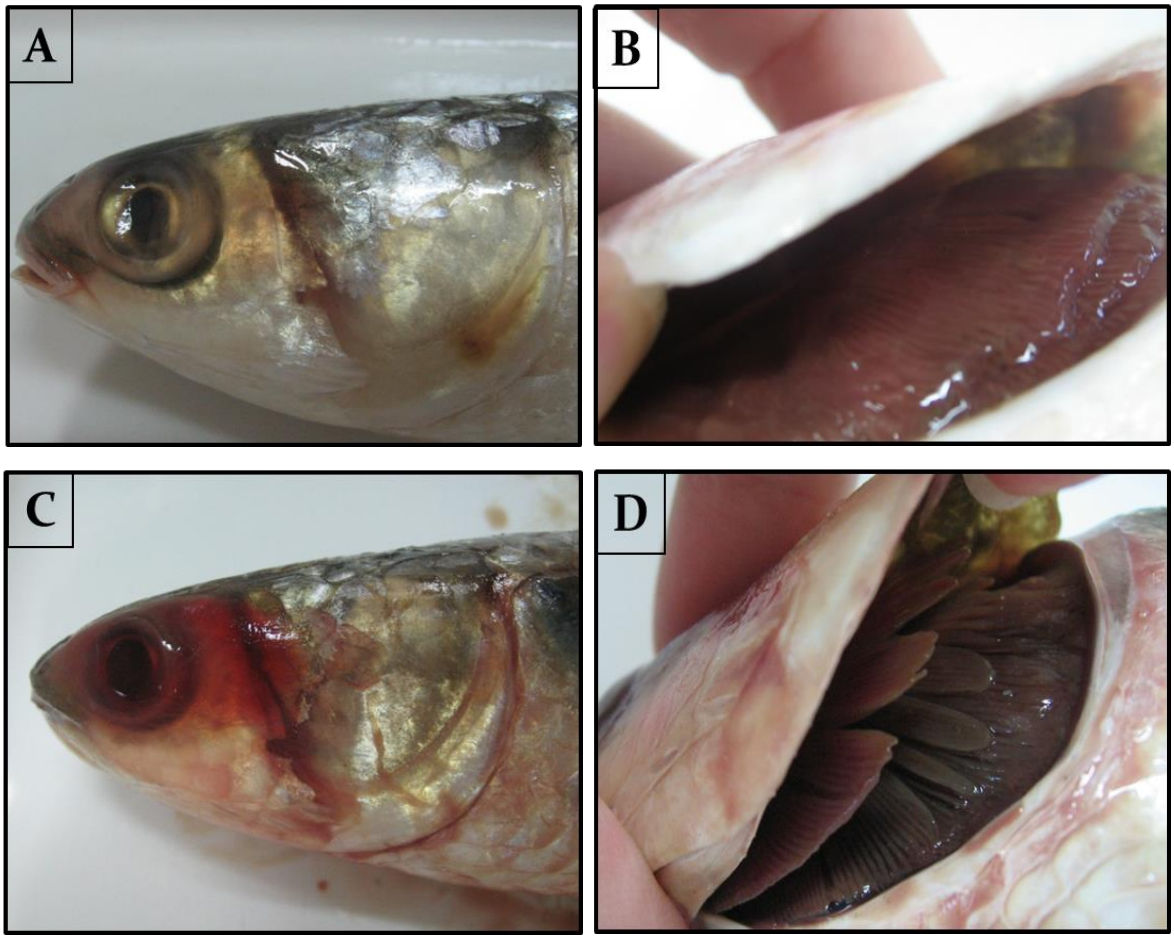


Figura: Área dos olhos e coloração das brânquias no 14º dia de estocagem da tainha a 0±1ºC (A e B) e 5±1ºC (C e D), respectivamente. Fonte: Arquivo pessoal

6.7 CROMATOGRAMA DOS PADRÕES DE NUCLEOTÍDEOS

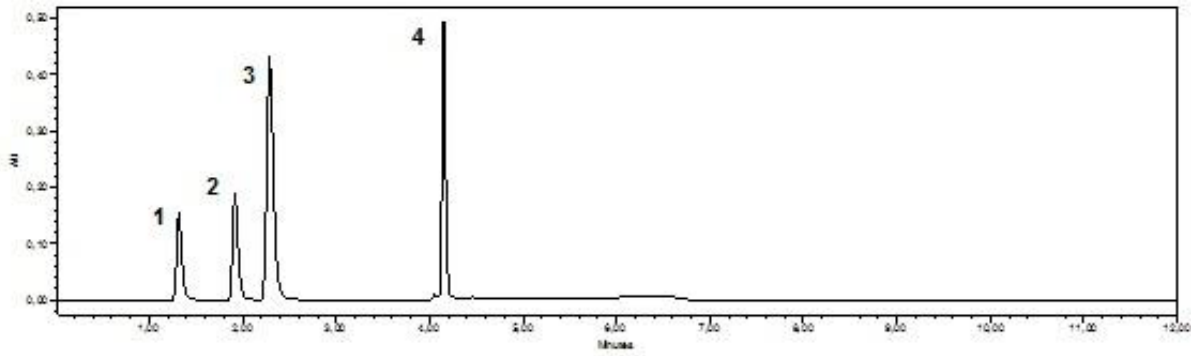


Figura: Cromatograma dos padrões de nucleotídeos:1 – inosina monofosfato, 2 – adenosina monofosfato, 3 – hipoxantina, 4 – inosina.

6.8 CROMATOGRAMA DOS PADRÕES DE AMINAS BIOGÊNICAS

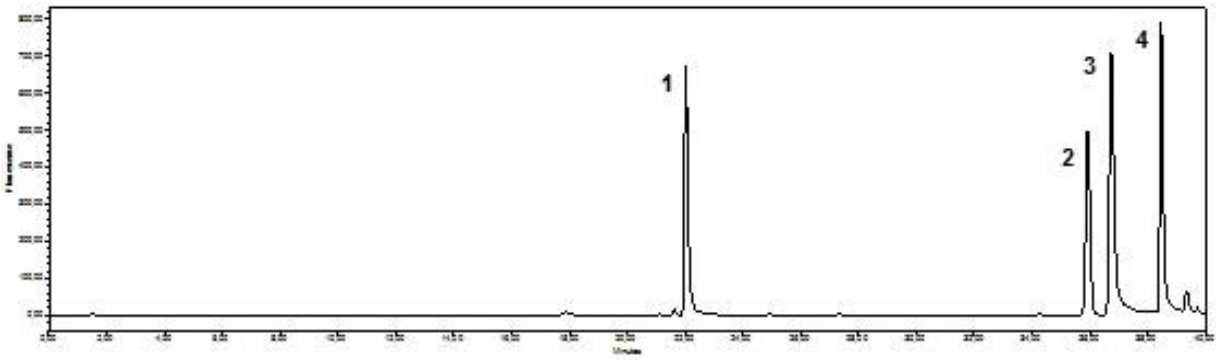


Figura: Cromatograma dos padrões de aminas biogênicas:1 – histamina, 2 – tiramina, 3 – putrescina.; 4 – cadaverina.

6.9 ARTIGO 4: CHEMICAL QUALITY INDICES FOR FRESHNESS EVALUATION OF FISH. Submitted to Reviews in Fisheries Science & Aquaculture.

Chemical quality indices for freshness evaluation of fish

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Abstract

The compounds formed in fish spoilage process have been frequently used to assess the quality of different species. Quality indices based mainly on concentration of the products of nucleotide degradation and biogenic amines content have been proposed. However, these indicators must be calculated considering intrinsic and extrinsic factors that may affect both, biogenic amine formation and nucleotide degradation rate and pattern. In relation to the analytical methods, regardless the methodology chosen, the extraction stage is critical. High performance liquid chromatography is the technique indicated for both, biogenic amine analysis and determination of nucleotide degradation products.

Keywords: analytical methods, biogenic amines, HPLC, quality index, nucleotide degradation products.

1. Introduction

After fish death, there is a gradual loss of freshness due to a series of *post mortem* enzymatic and bacterial reactions that determine the emergence of undesired odors and flavors due to the formation of organic compounds and the development of microorganisms that can be toxically hazardous for the consumer (Gram and Dalgaard, 2002; Andrade et al., 2012; Cardozo et al., 2013; Rodrigues et al., 2013).

The Brazilian law, through Decree no 30.691 (Brasil, 1952) and Ordinance no 185 (Brasil, 1997), establishes limits for fish freshness evaluation based on total volatile base content, tertiary volatile base content, internal and external flesh pH, hydrogen sulfide and histamine content for species of the *Scombridae*, *Scombresocidae*, *Clupeidae*, *Coryphaenidae* and *Pomatomidae* families. However it does not take into account other metabolites formed during fish spoilage process that have been used for some time as quality indices, such as adenosine triphosphate degradation products and presence of other biogenic amines (Carmo et al., 2010; Monteiro et al., 2010; Silva et al., 2011, Andrade et al., 2012; Rodrigues et al., 2013).

Assessing fish preservation based on official procedures has been considered a controversial issue because of the variety of species with individual peculiarities due to physiological aspects and capture methods.

Therefore, the objective of the present review is to conduct a study on quality index data, in order to support future analytical studies that contribute to effectively assess fish preservation, considering the physiological peculiarities of each species based on the concentration of biogenic amines and adenosine triphosphate degradation products highlighting the main analytical methods for the determination of those parameters.

2. Biogenic amines

Biogenic amines are organic compounds with basic character and low molecular weight formed by decarboxylation reactions of precursor amino acids ou by amination and deamination of aldehydes and ketones and synthesized in the metabolism of plants, animals and microorganisms (Brink et al., 1990; Bardócz, 1995; Sillas Santos, 1996; Rodriguez et al., 2014).

Although considered endogenous in some foods, and present in low concentrations, they are usually formed as a result of bacterial action associated to the availability of free amino acids, conditions that favor bacterial growth and enzyme production (Sillas Santos, 1996; Silva et al., 2011; Cardozo et al., 2013). The amount and type of biogenic amine formed depend on food composition and the type of microorganism present in the matrix (Brink et al., 1990).

Numerous bacterial genera present in food such as *Bacillus*, *Citrobacter*, *Clostridium*, *Klebsiella*, *Escherichia*, *Proteus*, *Pseudomonas*, *Salmonella*, *Shigella*, *Photobacterium*, *Lactobacillus*, *Pediococcus* and *Streptococcus* have amino acid decarboxylase capability. These microorganisms can be naturally present in food, intentionally added or introduced by contamination, as occurs by inadequate handling before, during or after food processing. The species *Morganella morganii*, certain *Klebsiella pneumoniae* strains and some of *Hafnia alvei* are prolific histamine producers, with particular importance in fish (Brink et al., 1990; Sillas Santos, 1996; Carmo et al., 2010; Lee et al., 2012).

Fish is highly susceptible to biogenic amine formation, especially histamine, putrescine, cadaverine and tyramine (Veciana-Nógues et al., 1997; Bunka et al., 2012; Hu et al., 2012). In general, high levels of biogenic amines found in seafood are caused by inadequate preservation with consequent microbial decarboxylation of amino acids (Hu et al.,

2012). In addition, some species, particularly those belonging to the *Scombridae* family, such as tuna fish and bonito and those of the *Clupeidae* family such as sardines, present high levels of free histamine in the muscle (Lehane and Olley, 2000; Oliveira et al., 2004; Kim et al., 2009; Lee et al., 2012). Histamine can be catabolized through two routes in fish muscle: by amino acid deamination forming urocanic acid, which is the main route in normal physiological conditions or by decarboxylation forming histamine, which is important route in cases of bacterial contamination (Sillas Santos, 1996; Lehane and Olley, 2000).

The content of biogenic amines present in fish can vary according to the season of the year, genetics, environment, food, sex, physiological stage, storage period and sampled tissue (Veciana-Nógues et al., 1997; Lee et al., 2012), and formation is directly influenced by the storage temperature (Silveira et al., 2001; Carmo et al., 2010).

The presence of biogenic amines in food, besides being a health problem due to its physiological and toxical effects (Önal, 2007), can be used as quality index, once they are formed by bacterial activity and are resistant to thermal treatment, thus reflecting thequality of the raw material and the hygienic conditions of food processing (Veciana-Nógues et al., 1997; Park et al., 2010; Sagratini et al., 2012).

2.1 Biogenic amine index

Biogenic amines are naturally present in very low levels in fresh fish and the presence of high amounts of these compounds is associated to bacterial degradation (Özogul and Özogul, 2006; Šimat and Dalgaard, 2011; Cunha et al., 2013). Assessing biogenic amine presence is important not only from a toxicological point of view but because these substances can be used as indicators of food degree of freshness or spoilage (Alberto et al.,

2002; Özogul and Özogul, 2006; Önal, 2007; Park et al., 2010; Sagratini et al., 2012; Silva et al., 2013).

Mietz and Karmas (1977), studying canned tuna to estimate the degree of freshness of the fish prior to processing, observed that on the spoiled samples, the putrescine, cadaverine and histamine content increased , while spermine and spermidine decreased compared to good quality food samples. Therefore, they suggested a Chemical Quality Index (QI) based on the concentration of the following biogenic amines:

$$Quality\ Index\ (QI) = \frac{histamine + putrescine + cadaverine}{spermine + spermidine}$$

The authors observed that the quality index increased when the sensory scoring of the canned product decreased. Thus, they suggested that a product with QI below 1 would have been processed with a first quality raw material, while those with values above 10, would indicate a raw material with very poor microbiological quality.

Veciana-Nóguez et al. (1997) assessing tuna stored at 0, 8 and 20ºC for 21, 9 and 4 days respectively, concluded that spermidine and spermine contents are not indicators of quality loss and that significant alterations in histamine, putrescine, cadaverine and tyramine concentrations during the studied period at the three storage temperatures were important to assess the spoilage process. In addition, they observed that sensory rejection of samples kept at 8ºC occurred on the 5th storage day although the QI value of 10 proposed by Mietz and Karmas (1977) had not been attained. These results support the proposal of a Biogenic Amine Index (BAI) based on the sum of histamine, cadaverine, putrescine and tyramine levels considering values below 50 mg.kg^{-1} as indicative of good quality food.

Křížek et al. (2002) found that the index proposed by Mietz and Karmas (1977) has little application to assess the quality of carp (*Cyprinus carpio*) stored in non-hermetic

packages kept at 3 and 15ºC for 13 and 4 days, respectively, due to non-occurrence of spermine concentration decline. Putrescine content presented the best correlation with the sensory quality of the meat. Based on these results, the authors observed that good quality samples presented putrescine content up to 10 $mg.kg^{-1}$, acceptable quality between 10-20 $mg.kg^{-1}$ and undesirable quality above 20 $mg.kg^{-1}$. Histamine and cadaverine formation kinetics was similar to putrescine formation kinetics, however histamine content increase was observed only in evidently spoiled samples. They considered, then, that the sum of putrescine and cadaverine contents might be used to assess carp quality.

Özogul and Özogul (2006) assessed biogenic amine concentration in sardines (*Sardina pilchardus*) kept at 4ºC in air, packed in modified atmosphere (60% CO_2 and 40% N_2) and under vacuum. From QI calculation proposed by Mietz and Karmas (1977) and BAI suggested by Veciana-Nógues et al. (1997), they observed that both indices increased as a function of storage time and shower good correlation with the sensory alterations of the samples. The QI 10 established as acceptance limit was attained in 4, 8 and 12 storage days in samples kept in the air, vacum packed and packed under controlled atmosphere, respectively, times when the samples were sensory rejected. The BAI presented good correlation with fish sensory quality, however, according to the authors, establishing acceptance limits for samples packed in vacum and modified atmosphere is still needed.

Bakar et al. (2010) studied biogenic amine content in barramundi (*Lates calcarifer*) stored at 0ºC and 4ºC for 15 days and calculared QI and BAI according to the formulas proposed by Mietz and Karmas (1977) and Veciana-Nógues et al. (1997). The results showed that both indices increased with storage time and therefore can be used to determine the degree of spoilage of this species.

Other freshness indices have been pointed out for different fish species based on the correlation of biogenic amine increase with storage period, such as cadaverine concentration

in salmon (*Salmo gairdneri*) (Yamanaka et al., 1989), cadaverine and agmatine content in smooth weakfish (Ruiz-Capillas and Moral, 2001) and putrescine and cadaverine content in trouts of the *Oncorhynchus keta* species (Rezaei et al., 2007; Rodrigues et al., 2013).

3. ATP degradation

Adenosine triphosphate is a high energy molecule that enables to keep actin and myosin filaments separated in the muscle. In living organisms ATP is regenerated from adenosine diphosphate (ADP) through oxidative phosphorylation. After the death of the animal, the cells continue, after a certain period of time, their normal physiological processes. As oxygen content decreases and creatinine phosphate reserve is consumed, ATP regeneration ends and ATP is rapidly degraded by a series of dephosphorylation and deamination reactions to different compounds (Ocaño-Higuera et al., 2009; Song et al., 2012).

ATP is thus converted, by dephosphorylation reactions initially to adenosine diphosphate (ADP) and then to adenosine monophosphate (AMP). AMP is in turn deaminated to inosine monophosphate (IMP) which degrades to inosine (HxR) and hypoxanthine (Hx). Several studies propose an alternative mechanism for marine invertebrates that considers a sequence of dephosphorilations to adenosine (Ade), which then degrades to HxR and Hx and, in some cases can form xanthine and uric acid (Saito et al., 1959; Venugopal, 2002).

Generally, ATP degradation occurs in a similar way on most marine species, however the degradation rate and pattern vary as a function of the specimen species, muscle type and biologic condition (sex, gonadal status), season of the year, water temperature, capture method and stress conditions during capture, handling and storage (Özyurt et a., 2007; Morkore et al.; 2010).

Gram and Huss (1996) highlight that usually, after the fish death, the ATP molecules rapidly hydrolyze to IMP by the action of endogenous amines. The subsequent degradation of IMP to HxR and Hx is slower and with the participation of autolytic and microbial enzymes. Therefore, IMP is accumulated in the initial degradation stage and an increase of Hx and HxR levels is observed with time and consequent quality loss. IMP is the main responsible for the definition of fresh fish odor and flavor, while Hx has a direct effect on the bitter taste of spoiled fish. Therefore, the content of ATP degradation metabolites has been widely used as biological indicators of fish freshness degree.

3.1 Quality indices based on ATP degradation

Saito et al. (1959) were the first authors to propose a quality index based on the concentration of different nucleotides, to assess fish freshness degree, defined as K value calculared by the formula:

$$K\ (\%) = \frac{HxR + Hx}{ATP + ADP + AMP + IMP + HxR + Hx}\ x\ 100$$

From the application of the K value, Saito et al. (1959) obtained values that allowed classifying some fish comercial species as:

- K < 20%: very fresh fish, suitable to be consumed raw.
- 20 < K < 40%: fish considered fresh to be consumed after cooking.
- K > 40%: fish inadequate for consumption.

As the passage of ATP to IMP is fast, Karube et al. (1984) modified K value and suggested a new calculation (Ki) defined as:

$$Ki\ (\%) = \frac{HxR + Hx}{IMP + HxR + Hx}\ x\ 100$$

Those authors highlighted that, for some species, ATP, AMP and IMP concentrations remain more or less constant up to two weeks. In this case, they should be considered for freshness evaluation.

Burns et al. (1985), studying samples of cod, mackerel and crab, proposed the G value based on the accumulation and/or degradation of Hx, IMP, AMP and HxR, according to the following formula:

$$G(\%) = \frac{Hx + HxR}{HxR + IMP + AMP}\ x\ 100$$

According to these authors, the G value is based on Hx accumulation also reflecting the dissapearance of IMP, AMP and HxR.

Luong et al. (1992) highlight that, for some species, K and Ki values do not adequately reflect the alterations that occur, because they rapidly increase and then, they remain more or less constant. This is caused by a rapid accumulation of HxR and Hx. For these species, those authors proposed the value H based on Hx concentration considered as a good indicator of fish freshness under both points of view, physiological and sensory, as a function of the characteristic bitter taste of spoiled fish:

$$H(\%) = \frac{Hx}{IMP + Hx + HxR}\ x\ 100$$

Lakshmanan et al. (1996) when assessing mullet (*Liza corsula*) and pearlspot (*Etroplus suratensis*) stored at room temperature and in ice reported K values different from those proposed by Saito et al. (1959). At room temperature, both species remained adequate for consumption up to 9-hour storage, when K value attained 50% and rejection occurred after 12-hour storage, with K values of 61.52 for mullet and 67.76 for pearlspot. Mullet and pearlspot ice stored samples were classified as first quality after 4 and 8 storage days with K values of 29.8% and 23.5% respectively, good with K values of 50% after 8 and 13 storage days and rejected after 12 and 14 storage days with K values of 70.59% and 54.94%, respectively. Özogul et al. (2011) suggested a K value of 80% as acceptance limit for sole (*Solea sole*) samples after 16-18 storage days.

In canned sardines (*Sardinops sagax caerulea*) of three different brands, Vázquez-Ortiz et al. (1997), aiming at evaluating the quality of the raw material before thermal processing, once it does not interfere on nucleotide content observed a K value variation of 34.7-56.3 indicating the use of a raw material of poor sensory quality. Similar results were described by Uriarte-Montoya et al. (2010) which observed an increase of K value throughout the canning process stages of this sardine species, from 14.1% to 22.8%. Both authors concluded that this index may be useful for freshness assessment of the fish used on the preparation of canned preserves.

Özogul et al. (2000) noted that herring (*Clupea harengus*) stored in ice and in modified atmosphere (60% CO_2 and 40% N_2) presented initial K values of 29% and 32% respectively, indicating poor quality raw material. In that study, the authors also verified that hypoxanthine increased more rapidly in ice stored fish than in fish stored in modified atmosphere, indicating that the presence of carbon dioxide (CO_2) influences Hx accumulation. Similar results were obtained by Özogul and Özogul (2002) when they analyzed trouts (*Oncorhynchus mykiss*) stored in modified atmosphere (40%CO_2, 30%O_2 and 30% N_2) and in

ice. In addition to K value, the authors calculated Ki and H values and observed that H value increased at a smaller rate than the other indices. They also reported that, although CO_2 presence influences Hx content throughout the storage period, the concentration of this gas did not affect K, Ki and H values.

Özogul et al. (2006a) calculated K, Ki, H and G values for European seabass (*Dicentrarchus labrax*) stored at 4ºC and in ice and observed continuous increase of these indices throughout the storage period. Corroborating findings of Özogul and Özogul (2002) found that H value slowly increased not presenting a significant alteration during the initial eight storage days due to the constant increase of Hx concentration. In addition, the authors reported that a rapid increase of K, Ki and G values occurred due to the accentuated drop of IMP and no significant difference was observed among those values. Similar results were observed by Özogul et al. (2006b) when assessing eel (*Anguilla anguilla*) and Özogul et al. (2008) when assessing white grouper (*Epinephelus aeneus*). For eles, the authors also calculared the P value and observed that the lowes value obtained was G value. The authors attributed acceptance limits for K, Ki, H and G of 81%, 84%, 39% and 137%, respectively in grouper.

Huidobro et al. (2001) assessing gutted and entire giltheads (*Sparus aurata*) obtained by diferente sacrifice methods (immersion in water and ice, asphyxiation, anestesia with subsequent percussion and percussion) observed da ATP degradation products could not be used separately as indicators of this species freshness, however the relationship expressed as K value presented good correlation with freshness status, with no significant differences among slaughter methods and *post-mortem* treatment. The K value of 20% was reached after seven storage days, presenting a slower evolution when compared to other species, reaching 50-60% only after 25 storage days, when the sensory and microbiologic evaluations showed values compatible with quality loss. The authors compared K and Ki values and noted that Ki

differed from K during the refrigerated storage, due to a rapid ATP degradation to IMP in this species, which allowed to infer that this value did not add relevant analytical information for quality assessment.

In ice stored matrinxã (*Brycon cephalus*) specimens, Batista et al. (2004) observed that during the initial storage days, there was no significant variation of K index (2.01 to 3.29%) and that, after 16 days, the K value attained 19.56 maintaining a significant correlation with sensory, physicochemical and bacteriological parameters and the fish was considered adequate for consumption, according to the classification proposed by Saito et al. (1959). After 23 storage days K value attained an average of 28.87%, maintaining freshness characteristics.

Siripatrawan et al. (2009), studying mollusks of the *Haliotis asinina* species noted that ATP alone could not be used as a freshness index, due to its rapid convertion to IMP. The authors observed Hx increase but did not detect inosine presence. They concluded that, for the studied species, K value is not a good indicator of degree of freshness.

4. Analytical methods

According to Önal (2007) the main applications of biogenic amine analysis refer to quality control of raw materials, intermediary and final products, monitoring of fermentation processes, process control, besides the technical-scientific aspects.

The complexity of the matrix to be analyzed, presence of interfering substances and low concentration are considered limiting factors in the analysis of these compounds in food (Shalaby, 1999; Alberto et al., 2002; Önal, 2007). The solvents more commonly used for the extraction of these compounds are trichloroacetic acid (Pacheco-Aguilar et al., 1998; Mendes et al., 1999; Shalaby, 1999; Ruiz-Capillas and Moral, 2001; Oliveira et al., 2004; Özogul et al.

2006a; Özogul et al., 2006b; Özogul and Özogul, 2006; Rezaei et al., 2007; Özogul et al., 2008; Anderson, 2008; Bakar et al., 2010), perchloric aid (Yamanaka et al., 1989; Křížek et al., 2002; Shakila et al., 2003; Dadáková et al., 2009; Kim et al., 2009) and methanol (Kim et al., 2001; Du et al., 2002).

Several analytical methods have been developed to assess biogenic amines in different foodstuffs. Spectrofluorimetry, thin layer chromatography, high performance liquid chromatography (HPLC), gas chromatography, capillary electrophoresis, and polymerase chain reaction (PCR) are mentioned as the more relevant from the analytical point of view (Shalaby, 1999; Lapa-Guimarães and Pickova, 2004; Önal, 2007).

Spectrofluorimetry has been used to determine amines individually (Andrade et al., 2012) and is considered by the AOAC as the official method in the USA and adopted as such in Brazil for the quantitative analysis of histamine in fish (AOAC, 2002; Brasil, 1997). However, recently high performance liquid chromatography (HPLC) became the official method in Brazil for the determination of histamine and other biogenic amines (cadaverine, putrescine, spermidine, spermine) in fish through Normative Instruction no 25 (Brasil, 2011). The technique involves acid extraction of the amines, pre-column off-line derivatization with dansyl chloride in alkaline pH, followed by separation and quantification by HPLC with elution gradient and ultraviolet detection. According to Park et al. (2010) HPLC is a selective and sensible technique and the most frequently used for biogenic amine determination due to its high resolution.

Thin layer chromatography is a simple, fast and low-cost semi-quantitative method for the separation and estimation of biogenic amines in food (Shalaby, 1999; Carmo et al., 2010; Andrade et al., 2010; Lapa-Guimarães and Pickova, 2004; Önal, 2007; Monteiro et al., 2010; Andrade et al., 2012; Rodrigues et al., 2012) used in routinairy quality control in industries and warehouses.

Due to the lack of chromophores and fluorescent properties of most biogenic amines, chemical derivatization is performed to increase the sensitivity in the determination of these compounds (Alberto et al., 2002; Park et al., 2010). Önal (2007) highlights that the analytical procedures adopted are usually based on the formation of fluorescent derivatives with different derivatization agents such as dansyl chloride, benzoyl chloride, fluorescein, 9-fluorenylmethyl chloroformate, naphtalene-2,3 dicarboxaldehyde and o-phtaldehyde (OPA). The use of the derivatization agent 6- aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) is a major breakthrough in the detection of biogenic amines because, among other factors, it has great stability and sensitivity to fluorescent detection (Martínez et al., 2000; Ordóñez et al., 2013).

Many methods have been reported for the analysis of ATP and its degradation products, such as the use of cationic-ionic exchange columns (Saito et al. 1959), enzyme sensor system (Karube et al. 1984), capillary electrophoresis (Luong et al., 1992) and high performance liquid chromatography (HPLC), the latter as the most frequently described in several studies (Burns et al., 1985; Lakshmanan et al., 1996; Vázquez-Ortiz et al., 1996; Özogul et al., 2000; Huidobro et al., 2001; Özogul et al., 2008; Siripatrawan et al., 2009; Uriarte-Montoya et al., 2010; Özogul et al., 2011). Perchloric acid is the reagent more frequently used in the extraction stage. Compound separation is usually performed in reverse phase columns using phosphate buffers as mobile phase or by ion-pair methods. Organic solvents such as methanol and acetonitrile can be used to reduce the run time (Özogul et al., 2000).

5. Conclusion

Quality indices based on the concentration of ATP degradation products and level of biogenic amines are widely used to assess fish freshness, because they present good correlation with the changes that occur during the storage period. However, aspects related to fish species, type of sampled tissue, stress suffered by the fish during capture, storage temperature, among other factors must be considered when establishing values to be reliably used.

In relation to the analytical methods, the extraction phase is one of the most important stages because of the elimination of interfering substances. The methodology commonly used for both, biogenic amine and ATP degradation product detection is high performance liquid chromatography, mainly because it allows the rapid and simultaneous detection of more than one compound in the same sample and analysis.

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6.10 COMPROVANTE DE SUBMISSÃO DO ARTIGO AO PERIÓDICO REVIEWS IN FISHERIES SCIENCE & AQUACULTURE

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6.11 ARTIGO 5: DETERMINAÇÃO DOS PRODUTOS DA DEGRADAÇÃO DA ADENOSINA TRIFOSFATO EM AMOSTRAS DE PESCADO POR CROMATOGRAFIA LÍQUIDA DE ALTA EFICIÊNCIA. Apresentado no Congresso no Congresso Higienistas de Alimentos 2013 e publicado na Revista Higiene Alimentar, v. 27, p. 1270-1273, 2013.

DETERMINAÇÃO DOS PRODUTOS DA DEGRADAÇÃO DA ADENOSINA TRIFOSFATO EM AMOSTRAS DE PESCADO POR CROMATOGRAFIA LÍQUIDA DE ALTA EFICIÊNCIA

DETERMINATION OF DEGRADATION PRODUCTS OF ADENOSINE TRIPHOSPHATE IN SAMPLES OF FISH USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Palavras-chave: separação, nucleotídeos, pescado, CLAE.

Introdução

A degradação da Adenosina Trifosfato (ATP) pode refletir as primeiras alterações no pescado antes do crescimento bacteriano. Este processo inicia-se logo após a morte por ação de enzimas presentes no músculo originando, em sequência, as substâncias ADP – adenosina difosfato, AMP – adenosina monofosfato, IMP – inosina monofosfato, HxR – inosina; Hx – hipoxantina; X – xantina e U – ácido úrico (SAITO et al., 1959; VECIANA-NOGUÉS et al., 1997; HUSS, 1999; VENUGOPAL, 2002).

A passagem de ATP a IMP é rápida, contudo, a transformação de IMP em Hx é mais demorada. Sendo assim, o IMP é acumulado na etapa inicial, fato que contribui para a definição do paladar do peixe, uma vez que este nucleotídeo é um importante componente relacionado ao sabor. Com o tempo, quando a qualidade do peixe decresce, verifica-se aumento dos níveis de hipoxantina e inosina (GRAM; HUSS, 1996; HUSS, 1999).A hipoxantina, formada pela decomposição autolítica de nucleotídeos ou por ação bacteriana, é um metabólito com efeito direto sobre o sabor amargo dos peixes em deterioração, portanto, seu conteúdo constitui um bom indicador do grau de frescor do peixe (GRAM; HUSS, 1996).Desta forma, o teor dos compostos formados a partir da degradação do ATP constitui um bom indicador da qualidade do pescado.

Diversos métodos têm sido descritos para análise dos produtos da degradação do ATP como colunas de troca cátion-iônica (SAITO et al. 1959), eletroforese capilar (LUONG et al., 1992) e biosensores (VENUGOPAL, 2002), porém a Cromatografia Líquida de Alta Eficiência (CLAE) é o método de eleição, pois além de separar todos os compostos, permite a quantificação dos mesmos, conforme descrito por diversos autores (RYDER et al., 1985;VECIANA-NOGUÉS et al., 1997;VÁZQUEZ-ORTIZ et al., 1997; ÖZOGUL et al., 2000; VALLS-DELGADO, 2000; SIRIPATRAWAN et al., 2009; SONG et al., 2012).

O objetivo do presente estudo foi a otimização de uma metodologia para separação e identificação dos produtos da degradação do ATP em amostras de peixe baseada na cromatografia líquida de alta eficiência em fase reversa.

Material e Métodos

A separação cromatográfica foi desenvolvida no cromatógrafo Waters® modelo Alliance® 2695 com detector de arranjo de fotodiodo Waters® 2996 utilizando-se uma coluna BDS Hypersil C_{18}, 2,4 µm, 100 x 4,6 mm Thermo® em forno a 30ºC.

A fase móvel empregada foi constituída de fase A (0,04 M KH_2PO_4 e 0,06 M K_2HPO_4) com pH 7,0 e fase B (acetonitrila) e o gradiente de eluição foi programado, conforme descrito na Tabela 1. A análise foi monitorada por absorção Ultra Violeta (UV) a 254 nm. O volume de injeção foi de 5 µL.

Para a análise dos compostos, foram utilizadas amostras de peixe adquiridas diretamente de barcos pesqueiros e mantidas sob refrigeração em gelo (0±1ºC). A extração dos nucleotídeos, conforme metodologia utilizada por Andrade et al. (2012), foi realizada através da homogeneização de partes da porção muscular do peixe em gral com pistilo e posterior pesagem em duplicata de uma alíquota de 50 mg em balança analítica Marte® modelo AY220. A esta amostra foi adicionado 1 mL de ácido perclórico ($HClO_4$) a 8% seguida da homogeneização em agitador ultrassom Branson® modelo 2210 por um período de 10 minutos. Posteriormente, adicionou-se 200 µL de hidróxido de potássio (KOH) 6M e homogeneizou-se em agitador mecânico Vortex-2 Geniedurante 20 segundos. Após, centrifugou-se a 9000 rpm por 3 minutos e o sobrenadante foi transferido para o vial com auxílio de um pipetador automático.

Os padrões adenosina monofosfato (pureza ≥ 99%), inosina monofosfato (pureza ≥ 98%), inosina (pureza ≥ 99%) e hipoxantina (pureza > 99%) da marca Sigma-Aldrich® foram preparados através da diluição de 10 mg em 0,5 mL de acetonitrila 1% e 0,5 mL de ácido fórmico 1%. Essa solução foi transferida para um balão volumétrico de 50 mL ao qual foi adicionado água Milli-Q até completar o volume.

Tabela 1: Gradiente de eluição da fase móvel utilizado para separação cromatográfica dos produtos da degradação da adenosina trifosfato

Tempo (min.)	Solução Tampão (A) (%)	Acetonitrila (B) (%)	Fluxo ($ml.min^{-1}$)
0	100	0	1,0
2	100	0	1,0
2,1	95	5	1,2
5	75	25	1,2
5,1	100	0	1,2
9,9	100	0	1,2
10	100	0	1,0

Resultados e Discussão

De acordo com o cromatograma apresentado na Figura 1, houve adequada separação dos padrões AMP, IMP, HxR e Hx com tempos de retenção em minutos de 2,18, 1,56, 5,85, 2,43, respectivamente. Nas amostras de peixe foi possível separar e identificar os quatro produtos da degradação da adenosina trisfosfato estudados, conforme o cromatograma apresentado na Figura 2. Sugere-se que os picos não identificados neste cromatograma sejam a adenosina trifosfato e outro produto de sua degradação.

O tempo total de corrida cromatográfica foi de 12 minutos e os compostos analisados foram resolvidos em seis minutos. Esse tempo foi inferior ao obtido por Ryder et al. (1985) e Vázquez-Ortiz et al. (1997) que ao trabalharem em condições isocráticas com uma fase móvel constituída apenas de solução tampão e uma coluna com partículas de 10µm, obtiveram resolução dos compostos em 16 e 13 minutos de corrida cromatográfica, respectivamente. Veciana-Nógues et al. (1996) utilizaram uma coluna com partículas de 5µm eobservaram que a adição de 30% de metanol à fase móvel após oito minutos de

corrida, diminui o tempo de resolução dos compostos de 22 para 12 minutos, porém esse período ainda é superior ao obtido nesse estudo.

A adição de acetonitrila na fase móvel após dois minutos de corrida, conforme sugerido por Özogul et al. (2000), associado ao uso de coluna com tamanho de partículas de 2,4µm, auxiliou na diminuição do tempo de retenção dos compostos analisados. O tempo restante foi necessário para retomada das condições cromatográficas iniciais.

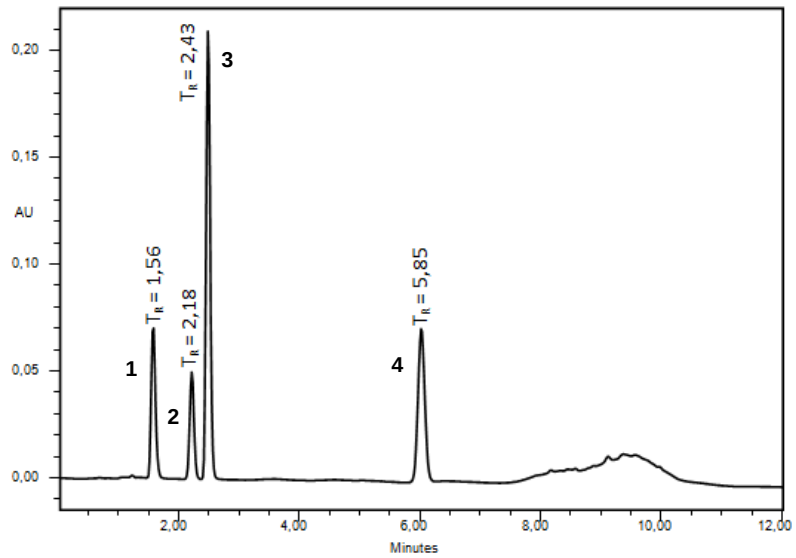

Figura 1: Cromatograma da mistura dos quatro padrões com os respectivos tempos de retenção: 1, inosina monofosfato; 2, adenosina monofosfato; 3, hipoxantina; 4, inosina.

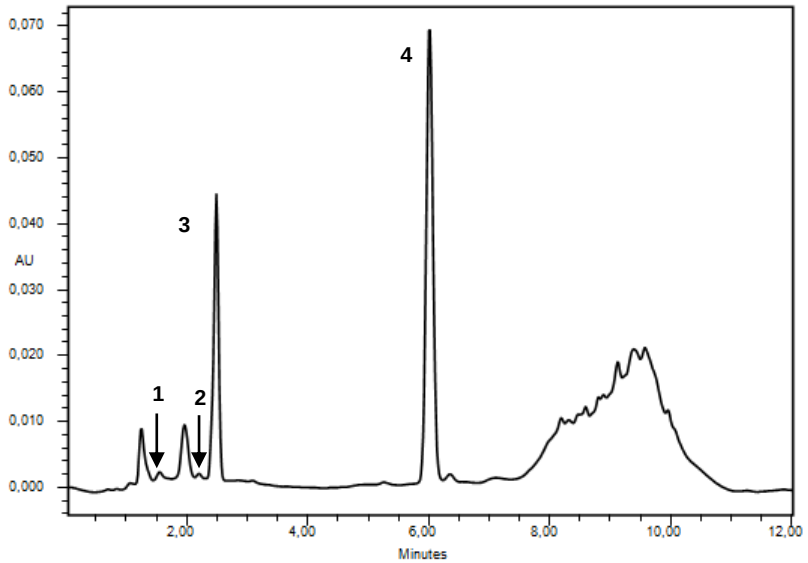

Figura 2: Cromatograma da amostra de peixe: 1, inosina monofosfato; 2, adenosina monofosfato; 3, hipoxantina; 4, inosina.

Conclusão

Através do método empregado foi possível separar e identificar os quatro produtos da degradação da adenosina trifosfato (AMP, IMP, HxR e Hx) analisados nesse estudo nas amostras de peixe. A utilização de coluna com partículas de 2,4 µm e a adição do solvente orgânico acetonitrila à fase móvel permitiram a diminuição no tempo de retenção das

substâncias avaliadas, com consequente diminuição do tempo e custo de análise e menor quantidade de resíduo da fase móvel descartado.

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