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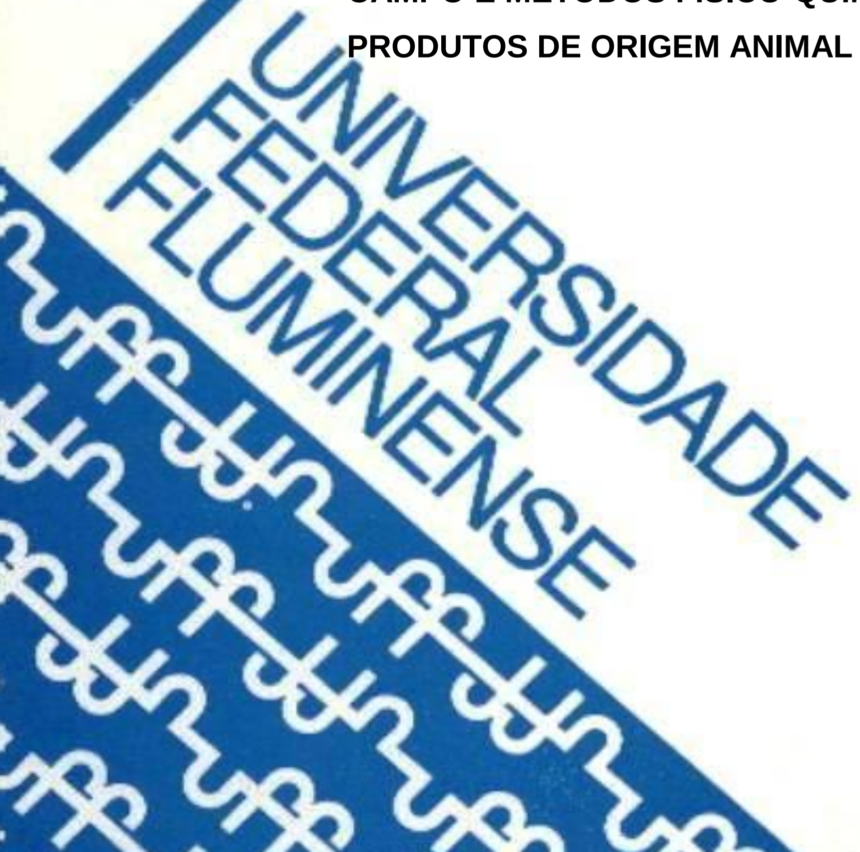
PROCESSAMENTO TECNOLÓGICO DE POA

ROBERTA DE OLIVEIRA RESENDE RIBEIRO

APLICAÇÃO DA RESSONÂNCIA MAGNÉTICA NUCLEAR DE BAIXO CAMPO E MÉTODOS FÍSICO-QUÍMICOS PARA ANÁLISE DE PRODUTOS DE ORIGEM ANIMAL



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

Orientadora: Profª Drª. ELIANE TEIXEIRA MÁRSICO

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“Aqueles que passam por nós, não vão sós, não nos deixam sós.
Deixam um pouco de si, levam um pouco de nós”

Antoine de Saint-Exupéry

RESUMO

Atualmente a indústria de alimentos está passando por um processo de transição no controle de qualidade, com tendência a adaptações e reciclagem nas análises laboratoriais visando rapidez, procedimentos não invasivos e não destrutivos, e que forneçam dados dos parâmetros de qualidade dos produtos em tempo real. Este recente panorama tende a resultar em uma forte integração de processos de controle de qualidade, gerando benefícios econômicos significativos. Desta forma, o presente estudo teve como objetivo verificar a aplicabilidade da Ressonância Magnética Nuclear de baixo campo (LF ^{1}H NMR) em duas diferentes matrizes (mel e bacalhau) para aplicação na indústria de alimentos; assim como demostrar através da utilização de métodos tradicionais de análises físico-químicos, a necessidade de um controle maior do mel durante o processamento, beneficiamento e armazenamento. O presente trabalho foi dividido em seis etapas: (1) Avaliar a aplicabilidade da LF ^{1}H NMR para classificação de méis brasileiros. Os resultados demonstraram que a técnica foi eficaz apresentando vantagens quando comparado a métodos de referência atualmente utilizados, que são custosos, demorados e de apresentam pouca sensibilidade analítica (Artigo I). (2) Detectar adulteração em méis utilizando a LF ^{1}H NMR. Observou-se que a técnica foi sensível para discriminar as amostras adulteradas, mesmo com baixas concentrações do adulterante (Artigo II). (3) Verificar os efeitos da temperatura de estocagem na qualidade do bacalhau do atlântico (*Gadus morhua*) utilizando a LF ^{1}H NMR. Observou-se que a ferramenta de análise é um instrumento sensível para observar as mudanças na mobilidade de água no interior do músculo do bacalhau do Atlântico durante o armazenamento, sendo uma alternativa útil para investigar as propriedades da água no bacalhau (Artigo III). (4) Verificar a influência do binômio tempo e temperatura no aumento do hidroximetilfurfural; os resultados determinaram que o teor de HMF aumentou significativamente com a exposição a temperaturas elevadas durante um longo período de tempo, sendo que esta conclusão contribui favoravelmente para o beneficiamento adequado do mel (Artigo IV). (5) Detectar fraudes em méis utilizando métodos físico-químicos de rotina; os resultados demostraram que apesar de detectar as fraudes, as técnicas apresentaram limitações, necessitando de maiores concentrações de adulterante para ser eficaz (Artigo V). Baseado nos dados obtidos nas etapas do trabalho concluiu-se que a técnica de LF ^{1}H NMR demonstrou ser uma ferramenta viável para utilização na indústria de processamento e classificação de diferentes matrizes alimentares. Apresentando vantagens claras quando comparado a métodos tradicionais de análise físico-químicas de alimentos, principalmente por se tratar de uma técnica rápida, não destrutiva e não invasiva. No entanto, o esclarecimento de algumas características do tempo de relaxação em produtos de origem animal ainda deve ser explorado no âmbito das pesquisas.

Palavras-chave: Mel; LF ^{1}H NMR, Bacalhau do atlântico (*Gadus morhua*); Hidroximetilfurfural; fraude; Classificação de méis.

ABSTRACT

Currently the food industry is undergoing a transition process in quality control, tending to adaptations and recycling in laboratory tests aiming fast, non-invasive and non-destructive procedures, and to provide data of the quality parameters of the products in real time. This recent overview tends to result in a strong process integration of quality control, generating significant economic benefits. Thus , the present study aimed to verify the applicability of Low Field Nuclear Magnetic Resonance (LF ^{1}H NMR) in two different matrices (honey and cod) for application in the food industry , as well as demonstrate through the use of traditional methods physicochemical analyzes , the need for greater control of honey during processing, processing and storage. This work was divided into six stages: (1) evaluate the applicability of LF ^{1}H NMR for classification of Brazilian honeys. The results showed that the technique was effective with advantages when compared to reference methods currently used, which are expensive, time consuming and have a low analytical sensitivity (Article I). (2) detect adulteration in honey using LF ^{1}H NMR . It was observed that the technique was sufficiently sensitive to discriminate against adulterated samples, even at low concentrations of the adulterant (Article II). (3) To investigate the effects of storage temperature on the quality of the Atlantic cod (*Gadus morhua*) using LF ^{1}H NMR. It was observed that the tool is a sensitive analytical tool to observe changes in the mobility of water into the Atlantic cod muscle during storage, as a useful alternative to investigate the properties of water in cod (article III). (4) Verify the combined influence of time and temperature on the increase of hydroxymethylfurfural, the results determined that the HMF content increased significantly with exposure to high temperatures for a long period of time, and this finding contributes favorably to the proper processing of honey (Article IV). (5) Detect fraud honeys using physicochemical methods of routine, the results showed that although detect fraud, technical limitations presented, requiring higher concentrations of adulterant to be effective (Article V) . Based on the data obtained in this research it was concluded that the technique of LF ^{1}H NMR showed to be a viable tool for use in processing industry and classification of different food matrices. Featuring clear advantages when compared to traditional methods of physicochemical analysis of food, mainly because it is a rapid, non-destructive and non-invasive technique. However, clarification of some characteristics of the relaxation time on products of animal origin should be further explored in the context of study.

Keywords: Honey, LF ^{1}H NMR, Atlantic cod (*Gadus morhua*); Hidroximetilfurfural; fraud; Classification of honeys .

SUMÁRIO

LISTA DE ILUSTRAÇÕES

1 INTRODUÇÃO

Tendo em vista a procura do mercado e a importância econômica que a venda de produtos de origem animal representa para as indústrias em todo o mundo (TSIRONI et al., 2009), destaca-se a relevância de estudos e avaliações que contribuam com dados sobre a características dos alimentos, assim como, a utilização de técnicas não convencionais e de interesse para indústrias, que avaliem estes resultados.

Nas últimas décadas, diversos métodos de controle de qualidade, em conjunto com a análise estatística multivariada, têm sido utilizados com sucesso para classificar o mel de diferentes regiões geográficas, assim como detectar adulteração e descrever características químicas deste alimento (CORDELLA et al., 2003, SERRANO et al., 2004 , PUSCAS et al., 2013). Entretanto, as características físico-químicas do mel ainda são pouco conhecidas, principalmente nas regiões tropicais, onde a flora apícola é bastante diversificada, associada às taxas elevadas de umidade e temperatura. É de fundamental importância a caracterização de méis visando à criação de padrões, segundo os fatores edafoclimáticos e florísticos da região, estabelecendo critérios comparativos nas análises e controlando possíveis fraudes desse produto (SODRÉ et al., 2007).

A apicultura é considerada uma atividade importante para o setor agropecuário em nível nacional. No ano de 2012, o Brasil exportou 12.615 toneladas de mel sendo considerado o 9º maior exportador mundial (BRASIL, 2013). Em adição aos aspectos econômicos, a apicultura brasileira reúne alguns requisitos que também a credenciam como uma atividade de elevado potencial de inclusão social, atendendo às características econômicas, sociais e ambientais (MOREIRA, 1996).

Entre os métodos analíticos utilizados para caracterizar alimentos, nas últimas décadas a LF ^{1}H NMR alcançou aceitação geral como uma ferramenta analítica em função de características não invasivas e de alta reprodutibilidade e sensibilidade, como demonstrado em uma ampla gama de aplicações (KAROUI et al., 2007, ALAM, ALAM, 2004). A LF ^{1}H NMR pode ser considerada uma técnica rápida para aplicações gerais, visando investigar a mobilidade de água nos materiais e alimentos (RUAN, CHEN, 2001; MARTINEZ et al., 2003, GUDJONSDOTTIR et al., 2011, CARNEIRO et al., 2013). Nas indústrias de processamento de alimentos, há um crescente e atual interesse na utilização de metodologias rápidas, não invasivas e não destrutivas. Sendo assim, técnicas e procedimentos demorados, que necessitem de extração, cominuição e/ou diluição, não são de interesse nessa área (AURSAND et al., 2008).

No entanto, não existe nenhum relato científico sobre a utilização da LF ^{1}H NMR para classificação de méis, não havendo relatos na literatura sobre a utilização para determinação de possíveis adulterações de méis. Com isto, a avaliação do potencial desta técnica para classificar méis de diferentes origens florais e diferenciar méis puros de méis adulterados, é uma proposta inovadora, com intuito de propor uma nova aplicação da técnica, visando maior controle da adulteração de méis, uma vez que o mel tornou-se alvo de adulteração com adoçantes mais baratos tanto no Brasil como em diversos países do mundo. O mel pode ser facilmente adulterado com vários adoçantes mais baratos, como o açúcar refinado da cana, xarope de milho hidrolisado e xarope de maltose, resultando em maiores lucros comerciais (RUIZ-MATUTE et al., 2010). Consequentemente, a discriminação entre o mel puro e o adulterado, tornou-se uma questão de relevância para produtores, varejistas e consumidores, bem como as autoridades reguladoras. Há uma crescente demanda por métodos analíticos adequados para garantir uma concorrência leal entre os produtores e proteger os consumidores de ações fraudulentas.

Ainda no âmbito do processamento de alimentos, destaca-se a salga, um dos processos mais antigos de conservação, com expressiva aplicação na área de pescado e derivados. O processo de adição de sal retarda, mas não inibe totalmente as alterações de degradação que ocorrem devido ao crescimento bacteriano durante o armazenamento. Durante a estocagem do pescado salgado, ocorrem alterações de degradação físico-químicas e alterações oriundas da ação de enzimas microbianas na estrutura proteica que interferem diretamente na mobilidade da água presente no alimento (interação água-proteína) (COULTATE, 2004).

Estudos demonstram correlação significativa entre parâmetros da técnica de Ressonância Magnética Nuclear (RMN) de Baixo Campo (LF ^{1}H NMR) e a dinâmica da água no tecido muscular de matrizes alimentares. Autores relatam fortes correlações entre as análises por RMN de baixo campo e as propriedades físico-químicas da água no músculo, tais como teor de umidade (ANDERSEN; RINNAN, 2002) e capacidade de retenção de água (JEPSEN et al., 1999; ERIKSON et al., 2004), alterações de pH muscular no *post mortem* (BERTRAM et al., 2000) e os efeitos do tempo de congelamento sobre a desnaturação de proteínas em pescado (STEEN; LAMBELET , 1997). Alguns artigos relatam que a LF ^{1}HNMR pode indicar a qualidade do pescado quando os mecanismos de deterioração afetam as propriedades da água e a estrutura dos músculos (proteínas) (ANDERSEN; RINNAN, 2002; BERTRAM; ANDERSEN, 2004; BERTRAM; ANDERSEN, 2007; AURSAND et al. 2009; GUDJÓNSDÓTTIR et al., 2011a; GUDJÓNSDÓTTIR et al., 2011b).

Deste modo, o presente estudo buscou trazer contribuições inéditas através da utilização de RMN de baixo campo de prótons de Hidrogênio (^{1}H), para obter dados sobre a mobilidade da água no mel, com intuito de diferenciar floradas e detectar possíveis fraudes, assim como, correlacionar os resultados dos tempos de relaxação transversal T_2 e seus exponenciais T_{21} e T_{22}, obtidos através da RMN, com os dados obtidos em análises físico-químicas que também avaliem a dinâmica da água (umidade, capacidade de retenção de água, atividade de água, pH e cor). Objetivou-se também estudar as modificações ocorridas nos tempos de relaxação transversal de bacalhau (*Gadus morhua*) submetidos ao processo de salga seca, em diferentes tempos de armazenamento de modo a avaliar a variação deste parâmetro durante a deterioração do pescado. Em paralelo, avaliaram-se as amostras por técnicas físico-químicas, objetivando a classificação dos méis brasileiros, detecção de possíveis fraudes e contribuição com informações úteis para o processamento do mel, através da avaliação da influência do binômio tempo/temperatura na quantidade de Hidroximetilfurfural.

2 FUNDAMENTAÇÃO TEÓRICA

Este tópico irá abordar a estrutura fundamental do mel de abelha (*Apis mellifera*) e do Bacalhau do Atlântico (*Gadus morhua*) e, como ambos podem ser alterados por processamentos e tratamentos. Para compreender os efeitos de diferentes métodos de processamento e armazenamento sobre a qualidade das matérias-primas estudadas nesta pesquisa, é necessário o conhecimento geral das características e das propriedades físico-químicas do mel de abelha e do Bacalhau do Atlântico. Nesse novo conceito aplica-se a técnica de RMN de baixo campo. Pesquisas realizadas em produtos de origem animal verificaram boa aplicabilidade desta técnica, na avaliação da distribuição da água na musculatura, assim como, sua correlação com análises físico-químicas tais como pH muscular, umidade e capacidade de retenção de água, ou seja, parâmetros que são influenciados tanto por fraudes, quanto pela deterioração que ocorrem durante o tempo de armazenamento (GUDJÓNSDÓTTIR et al., 2011).

2.1 ASPECTOS GERAIS SOBRE O MEL

O mel, por definição, é um produto natural de abelhas obtido a partir do néctar das flores (mel floral), de secreções de partes vivas das plantas ou de excreções de insetos sugadores de partes vivas das plantas (mel de melato) (CAMPOS, MODESTA; 2000). Trata-se de uma matriz muito complexa, com interferência de variáveis não controladas pelo homem, como clima, floração, presença de insetos sugadores e outros fatores. As abelhas, por sua vez, vão utilizar os recursos disponíveis como fonte de açúcar para elaborá-lo. O mel é constituído essencialmente de vários açúcares, predominantemente D-frutose e D-glicose, como também de outros componentes e substâncias como ácidos orgânicos, enzimas, e partículas sólidas coletadas pelas abelhas. A aparência do mel varia de quase incolor a marrom escuro. Pode ser fluido, viscoso, ou até mesmo sólido. Seu sabor e aroma variam de acordo com a origem da botânica.

O mel é considerado o produto apícola mais fácil de ser explorado, sendo também o mais conhecido e com maiores possibilidades de comercialização. Além de ser um alimento, é também utilizado em indústrias farmacêuticas e cosméticas, pelas suas conhecidas ações terapêuticas (FREITAS et al., 2004). É considerado um alimento muito rico e de elevado valor energético, consumido mundialmente e de extrema importância para a saúde do organismo humano quando puro, por apresentar diversas propriedades: antimicrobiana, regenerativa de tecidos e estimulantes (BIZZARIA et al, 2003).

2.2 PROPRIEDADES FÍSICAS DO MEL

A densidade do mel a 20°C varia de 1,38 a 1,45 g/cm^3. As diferenças das viscosidades entre as variedades diminuem com o aumento da temperatura. Acima de 40°C, o conteúdo de água alcança valores entre 16,4 e 20%, e não tem nenhum efeito significante na viscosidade, que depende da concentração de açúcar, conteúdo de água e temperatura, assim como do conteúdo de dextrina, trissacarídeos e proteínas (RYBAK-CHMIELEWSKA, 2004).

A duração da fase líquida do mel depende de muitos fatores extrínsecos e intrínsecos como conteúdo de água, relação D-frutose e D-glicose, relação D-frutose e não açúcares, e o conteúdo de dextrina. Altas concentrações de açúcar total e D-glicose ou baixa quantidade de dextrina favorecem a cristalização do mel. A temperatura é o principal fator ambiental envolvido nos mecanismos de cristalização. A formação de núcleos cristalinos na primeira fase de cristalização acontece entre 5° e 8°C. A taxa de crescimento dos cristais é mais alta a temperaturas entre 13° e 17°C. A temperatura de armazenamento não só afeta a fase líquida, mas também a maneira de cristalização, podendo ser formados cristais maiores em méis com menos núcleos cristalinos, que tendem a precipitar-se no fundo do recipiente, resultando em uma estrutura estratificada (RYBAK-CHMIELEWSKA, 2004).

2.3 COMPOSIÇÃO DO MEL

A composição do mel depende, principalmente, das fontes vegetais das quais é derivado e de diferentes fatores, como o tipo de solo, a espécie da abelha, o estado fisiológico da colônia, o estado de maturação do mel, as condições meteorológicas por ocasião da colheita, entre outros (PAMPLONA, 1989;

BOGANOV et al., 2002). A legislação brasileira define os padrões para o mel de abelhas melíferas, estabelecendo os requisitos mínimos de qualidade que o mel destinado ao consumo humano deve possuir: açúcares redutores (calculados como açúcar invertido), mínimo de $65g.100g^{-1}$, para o mel floral, e mínimo de $60g.100g^{-1}$, para o melato; umidade máxima de 20 $g.100g^{-1}$; sacarose aparente para o mel floral máxima de 6 $g.100g^{-1}$ e para o; sólidos insolúveis em água máximo de 0,1 $g.100g^{-1}$, exceto no mel prensado, que se tolera até 0,5 $g.100g^{-1}$, minerais (cinzas) máximo de 0,6 $g.100g^{-1}$ para o mel floral e máximo de 1,2g.100g-1 no melato. Além disso, o mel deve necessariamente apresentar grãos de pólen. Em relação à deterioração, o mel não deve ter indícios de fermentação, apresentar acidez máxima de 50 mil equivalentes por quilograma, atividade diastásica: como mínimo, 8 na escala de Göthe e teor de hidroximetilfurfural máximo de 60 $mg.kg^{-1}$ (BRASIL, 2000).

A composição exata de qualquer mel depende principalmente das fontes vegetais das quais é derivado, mas também do tempo, solo e outros fatores. O mel varia muito de uma região para outra, tanto em conteúdo polínico como em características físico-químicas, e isso é explicado pela possibilidade de ser originado de mais de 2500 tipos de flores de plantas diferentes (MONTENEGRO et al., 2003).

A D-glicose e a D-frutose podem originar-se do néctar ou melato e da hidrólise enzimática de sacarose e outros açúcares do mel. Rafinose e melecitose originam-se do néctar ou de melato. Outros di- e tri-sacarídeos são o resultado da ação de enzimas de mel. Mel também contém certa quantidade de dextrina (3 a 10%) (RYBAK-CHMIELEWSKA, 2004). Os ácidos orgânicos contribuem substancialmente ao sabor característico de mel enriquecendo e diversificando o sabor de variedades de mel (SIMPSON et al., 1975). Ácidos butírico, acético, fórmico, lático, succínico, fólico, málico, cítrico e glucônico foram identificados em mel, sendo os dois últimos os principais ácidos (PEREIRA, 2003). O ácido glucônico é o produto da oxidação catalítica específica de D-glucopiranose com glicose oxidase, uma flavoproteína enzimática do mel. A glucolactona, que é o resultado da oxidação, prontamente hidrolisa em ácido glucônico. O Potássio, magnésio, sódio, cálcio, fósforo, ferro, manganês, cobalto, cobre e alguns outros minerais têm sido identificados no mel. O potássio é o elemento principal no mel, excedendo o conteúdo dos outros minerais em várias ordens. Uma correlação alta também foi verificada entre os conteúdos de potássio e magnésio. Sais minerais, ácidos orgânicos, e aminoácidos em mel dissociado, fazem do mel um eletrólito.

Os pigmentos do mel pertencem às classes dos carotenóides, antocianinas e flavonas. Os níveis do carotenóides (g/100 g) em algumas variedades de mel variam de 1,49 a 183,07. O mel é pobre em vitaminas, apresentando poucas variedades contendo traços de vitaminas A, B2, C e B6. Aproximadamente 80 compostos aromáticos têm sido detectados em mel, incluindo ácidos carboxílicos, aldeídos, cetonas, álcoois, hidrocarbonetos, e fenóis, também contribuem para propriedades sensoriais do mel (BOGDANOV, 2000).

Há aproximadamente 175 mg de aminoácidos livres (de 27 a 875 mg) em 100 g de mel de néctar. Em variedades de mel de melato, o conteúdo comum de aminoácidos livres é 178 mg (de 54 a 269 mg). O principal aminoácido livre, é a prolina, que constitui cerca de 49 e 59% do conteúdo de aminoácido livre total do mel de néctar e melato, respectivamente. O mel contém várias enzimas específicas: invertase, amilase, glicose oxidase, catalase, e fosfatase.

2.4 SALGA SECA DO PESCADO

A utilização da salga como método de conservação do pescado é descrita há mais de quatro mil anos com registros desde as civilizações do antigo Egito e Mesopotâmia (FLANTRIN; MONTANARI, 1998). A salga é um processo físico-químico de difusão e osmose no qual se verifica a penetração do sal e a saída de água do músculo, com efeitos acentuados nas proteínas musculares. Com a salga há redução na solubilidade e capacidade de retenção de água dos tecidos. O sal extrai a água ao mesmo tempo em que penetra no tecido muscular do pescado, convertendo a água presente na musculatura em uma solução concentrada de cloreto de sódio. As membranas celulares são semipermeáveis. Quando há penetração de sal em quantidade suficiente (equilíbrio osmótico), as proteínas coaguláveis se estabilizam e o tecido se contrai por perda de água. Durante a salga ocorre desnaturação das proteínas do pescado, verificando-se o desdobramento destas em peptídeos e aminoácidos (FELLOWS, 2006).

Produtos com altos teores de sal apresentam o prazo de validade comercial estendidos sendo considerados de fácil conservação, apesar das possíveis alterações bioquímicas e enzimáticas que podem a longo tempo diminuir a qualidade e/ou até consolidar o processo de deterioração (OGAWA, 1999). Durante a estocagem podem ocorrer alterações de deterioração como mucosidade e crescimento de microrganismos (bactérias e fungos). A mucosidade é caracterizada pela coloração amarelada e alteração na textura. O crescimento bacteriano acarreta a produção de enzimas autolíticas exógenas que atuarão degradando proteínas e peptídeos, causando alterações físico-químicas e sensoriais (textura, aspecto, odor). Em regra, o processo de deterioração origina odores amoniacais e sulfídricos oriundos da degradação proteica (GONÇALVES, 2011). O peixe salgado e seco deve ser um produto elaborado com peixe limpo, eviscerado, com ou sem cabeça e convenientemente tratado pelo sal (cloreto de sódio), com nível de saturação mínima de 95% com ou sem aditivos, devidamente seco, podendo conter até 40% de umidade para as espécies consideradas gordas, tolerando-se 5% a mais de umidade para as espécies consideradas magras (BRASIL, 1988).

O *Gadus morhua* é peixe demersal pertencente à família *Gadidae* conhecido vulgarmente como bacalhau do atlântico é e comercializado como “bacalhau”. No Oceano Atlântico ocidental, o bacalhau pode ser encontrado para norte do Cabo Hatteras e na Carolina do Norte; no Atlântico oriental é encontrado a norte da Baía da Biscaia até ao Oceano Ártico, incluindo o Mar Báltico, o Mar do Norte, o Mar das Hébridas, áreas em redor da Islândia e Mar de Barents (JÓNSSON, 1992). O habitat estende-se desde a linha de costa até à plataforma continental (JÓNSSON, 1992). O Bacalhau do Atlântico é um dos produtos mais exportado da Islândia e da Noruega, sendo os principais mercados o Sul da Europa e América Latina (BOUDHRIOUA et al., 2009).

2.5 ÁGUA NA MUSCULATURA DO PESCADO

A água é o constituinte mais abundante encontrado na maioria dos alimentos, sendo responsável por aproximadamente 80% no tecido muscular de peixes magros, como o bacalhau. Alterações na quantidade e propriedades da água na musculatura do pescado, portanto, desempenha um papel fundamental nas possíveis alterações vinculadas à qualidade durante o processamento e armazenamento (MURRAY; BURT, 2001). O teor de água afeta vários parâmetros de qualidade, tais como a aparência, a textura e a estabilidade de armazenamento do peixe. A água é também o principal meio de crescimento de microrganismos, sendo influenciado diretamente pelas propriedades da água, tais como umidade e atividade da água. As proteínas miofibrilares são os principais componentes responsáveis pela ligação à água. As alterações que ocorrem nas proteínas musculares, durante a transformação, portanto, influenciam a distribuição de água no músculo. Desta forma, a dinâmica da agua no músculo influencia a qualidade, textura, propriedades sensoriais e a validade comercial do produto. Durante o processamento a estrutura proteica do pescado é alterada, afetando as características do produto final. Cozinhar e congelar são exemplos de etapas de processamento comuns que são conhecidos para desnaturar e causar agregação de proteínas e levam à perda de água a partir do músculo (JENSEN et al., 2003) . O conhecimento sobre a distribuição e comportamento da água no músculo, é importante para minimizar a perda de água durante o processamento e armazenagem, e melhorar o controle do processamento, assegurando a estabilidade do produto.

2.6 DISTRIBUIÇÃO DE ÁGUA NO MÚSCULO

A água em tecidos musculares pode, geralmente, ser dividida em três compartimentos. A água intimamente ligada a proteínas, que corresponde a apenas cerca de 5% da água que se encontra no músculo (SIKORSKI , 2001) . A água é ligada à proteína por interagir com os resíduos de aminoácidos hidrofílicos sobre a superfície da proteína e as moléculas de água adicionais são ligadas na zona circundante dos resíduos de aminoácidos das multicamadas hidrofílicas (SIKORSKI , 2001; ALMUTAWAH et al. , 2009). A maior parte da água da porção muscular pode ser encontrada no interior da estrutura do músculo e nas células musculares. Esta população é conhecida como a água ligada. De acordo com Ofstad e Hermansson (1997), esta água é mantida principalmente na porção superficial do músculo, e devido à tensão superficial gerada na musculatura, pode ser parcialmente removida durante o processamento. Finalmente uma pequena população de água livre pode ser encontrada no músculo. Esta água livre é encontrada entre as células do músculo e pode ser facilmente removida.

2.7 RESSONÂNCIA MAGNÉTICA NUCLEAR

Descoberta independentemente por Bloch (BLOCH et al., 1946) e Purcell (PURCELL et al., 1946) em 1946, a Ressonância Magnética Nuclear (RMN) é uma técnica rápida, não destrutiva e não invasiva que pode ser utilizada para análise de diversas matrizes, incluindo alimentos (RUAN, CHEN, 2001; MARTINEZ et al., 2003; GUDJONSDOTTIR et al., 2011). Os métodos baseados na RMN têm várias vantagens, tais como permitir medições relativamente rápidas, não destrutivas e não invasivas. No entanto, o uso da LF ^{1}H NMR no campo da ciência dos alimentos está longe de ser plenamente explorado, e muito conhecimento ainda está para ser adquirido (SÁNCHEZ-ALONSO et al., 2012).

As propriedades da NMR têm origem na interação entre um átomo em um campo magnético externo; de forma mais precisa, é um fenômeno em que partículas contendo momento angular e momentos magnéticos exibem um movimento de precessão quando estão sob ação de um campo magnético. Os principais átomos que compõem os tecidos musculares são: hidrogênio, oxigênio, carbono, fósforo, cálcio, flúor, sódio, potássio e nitrogênio. Estes átomos, exceto o hidrogênio, possuem no núcleo atômico prótons e nêutrons. Apesar de outros núcleos possuírem propriedades que permitam a utilização em RMN, o hidrogênio é o escolhido por três motivos básicos: é o mais abundante no corpo humano e nos tecidos animais; as características de RMN diferem bastante entre o hidrogênio presente no tecido normal e no tecido patológico, ou degradado no caso de alimentos e; o próton do hidrogênio possui o maior momento magnético e, portanto, a maior sensibilidade a RMN (HARRIS et al., 2001).

O átomo de hidrogênio, o mais simples da tabela periódica, possui como núcleo o próton, partículas carregadas positivamente, que possuem uma propriedade chamada de spin ou momento angular.

O spin representa o movimento de giro do próton em torno de seu próprio eixo. Para o próton de hidrogênio, o spin (I) pode ser +1/2 ou -1/2. Em núcleos com um número par de subpartículas de mesmo nome, (prótons ou nêutrons) os spins se cancelam mutuamente de modo que o núcleo do átomo não possui nenhum momento magnético total. No entanto, em certos átomos como 1H, 13C, 31P, 15N, 19F, 23Na, entre outros, o núcleo possui um spin total diferente de zero, o que gera um momento magnético. A técnica de RMN se aplica ao estudo de núcleos com momento magnético não nulo, ou seja, com spin $I \neq 0$. Estes núcleos se comportam como pequenos ímãs. A aplicação de um campo magnético afeta os níveis de energia de spin permitindo observar, em ressonância, os espectros resultantes das transições entre estes níveis. Quando o núcleo que possui um momento magnético é colocado num campo magnético estático, irá interagir com este campo resultando em um movimento giratório oscilante ou angular que é chamado de precessão nuclear. A frequência desta precessão do núcleo é chamada de frequência de Larmor ($\omega = \gamma\beta0$) (GIL; GERALDES; 1987). Onde: ω, é a frequência de precessão; $\beta0$, é a intensidade do campo magnético estático, e γ é denominada é a razão giromagnética . Cada núcleo possui um valor diferente para essa razão. Esta representa uma indicação da intensidade do magnetismo nuclear (RUAN; CHEN, 2001). O núcleo do átomo de hidrogênio (um próton) configura-se como o mais abundante núcleo dentro dos tecidos biológicos sendo, consequentemente, o átomo de maior importância em estudos de RMN na área da ciência dos alimentos (GUDJONSDOTTIR et al., 2011). A espectroscopia de RMN é hoje usada de forma generalizada por todos os que se dedicam a estudar direta ou indiretamente compostos ou fenômenos químicos. A espectroscopia de RMN é uma técnica analítica sofisticada e poderosa que tem aplicações em diferentes áreas. A diversidade de aplicações só foi possível devido ao contínuo desenvolvimento de diferentes técnicas que se baseiam no fenômeno de RMN. Este desenvolvimento permitiu que ao longo dos tempos cada técnica fosse otimizada de acordo com as necessidades da perspectiva da aplicação. A RMN pode ser utilizada tanto para a análise qualitativa como quantitativa e as suas aplicações vão desde a análise de compostos químicos simples a seres vivos intactos, de um modo não invasivo e não destrutivo. Os campos mais comuns de aplicação da RMN incluem: Análise estrutural aplicada à química e à biologia; Imagiologia médica: tomografia de ressonância magnética nuclear ou imagiologia de ressonância magnética nuclear (maior área de aplicação); Prospecção geofísica (por exemplo, de petróleo): geotomografia de ressonância magnética nuclear. A utilização da espectroscopia de RMN está muito associada à determinação da estrutura molecular em solução, mas os estudos efetuados em estado sólido são também frequentes. A RMN pode ser aplicada não só na elucidação estrutural de pequenas e médias moléculas, como também no estudo de macromoléculas, como proteínas ou ácidos nucleicos. Além de ser muito útil na elucidação da estrutura molecular, a RMN pode ser também usada para estudar processos dinâmicos de moléculas e para estudar interações/reações entre moléculas. As experiências mais comuns LF ^{1}H NMR baseiam-se na observação direta de núcleos de ^{1}H em solução. As características favoráveis deste núcleo (elevada abundância natural, elevada razão giromagnética) possibilitam medições de alta sensibilidade e baixo tempo de aquisição para este núcleo e são responsáveis pelo vasto número de aplicações desta técnica que vai desde compostos muito simples com baixo peso molecular até proteínas (HARRIS et al., 2001).

Este método possui interesse para a indústria de alimentos, pois possibilita avaliar parâmetros físico-químicos e sensoriais, visto a correlação da técnica com as análises de água e lipídeos (AZEREDO; COLNAGO, 2000). Além disto, destaca-se como um método rápido, econômico e não destrutivo (BELTON, 2003). Como exemplos da utilização de LF ^{1}H NMR em alimentos destacam-se a análise de óleo em sementes, o estudo da distribuição de água e óleo em alimentos, o estudo do estágio de maturação de frutas e vegetais e a caracterização da água na estrutura muscular de carnes e pescado (GUNASEKARAN, 2001). A aplicação da LF ^{1}H NMR em pescado permite relacionar o estado e a dinâmica da água para vários parâmetros tecnológicos, como: medidas de condições de armazenamento, de matérias-primas, de qualidade e de processamento. Além disso, a estrutura dos músculos pode ser estudada indiretamente (AURSAND et a., 2009). Como relatado anteriormente, a ressonância é um método no qual ocorre absorção de radiação eletromagnética por um núcleo atômico, colocado em um campo magnético estático (PANEPUCCI; TANNÚS, 2000). Os parâmetros de análise por LF ^{1}H NMR são os tempos de relaxação denominados T_1(longitudinal) e T_2 (transversal), que dependem da mobilidade molecular da amostra. Sendo assim, populações diferentes de água nos tecidos podem ser estudadas porque os prótons em ambientes diferentes apresentam diferentes propriedades de relaxamento (AURSAND et al., 2009). T_1 e T_2 representam o tempo de retorno da amostra ao seu estado inicial, quando a excitação da mesma por um campo magnético é finalizada (GIL, 1987). Este método se aplica a núcleos de ^{1}H de espécies químicas no estado líquido mesmo que estejam dispersos em amostras sólidas (PRESTES et al., 2007). Quando os átomos de hidrogênio estão dispersos em uma matriz sólida, como, por exemplo, em um tecido muscular, o tempo de decaimento de T_2 (tempo de relaxação transversal) no tecido muscular é multiexponencial, indicando a existência de diferentes “populações” de água no tecido (RUAN; CHEN, 2001; COPE, 1969). Nos últimos anos, investigações no tempo de relaxação T_2 têm apoiado esta afirmação, tanto para pescado, quanto para outras carnes (BERTRAM, ANDERSEN, 2004; BERTRAM et al., 2002; LAKSHMANAN et al., 2007; GUDJÓNSDÓTTIR et al., 2011; AURSAND et al., 2008). Em estudos de LF ^{1}H NMR, esta água tem sido relatada com relaxação entre 1-10ms, e tem sido referida como o componente de relaxamento T_{2b} (BERTRAM et al., 2002). A quantidade de água ligada muda muito pouco, e são muito resistentes aos processos de *rigor mortis*, congelamento e cocção (DAMODARAN et al., 2010). No “segundo” compartimento encontra-se a água chamada de água de revestimento. Esta sim pode ser afetada pela conversão do músculo em carne, congelamento e cocção, podendo ser perdida ou não com o gotejamento (OFFER, 1988). Esta fração é citada em estudos de LF ^{1}H NMR com carnes e pescado, como T_{21}, sendo encontrada em tempos de relaxação que variam aproximadamente entre 40 até cerca de 80-100ms (BERTRAM, ANDERSEN, 2004; BERTRAM et al., 2002). O terceiro compartimento é onde se encontra a “água livre", que se mantêm na superfície do alimento por ligações fracas e é descrita em LF ^{1}H NMR de baixo campo como T_{22}, com tempo de relaxação próximo de 100 e até 400ms (BERTRAM et al., 2002). A água de revestimento e a água livre correspondem a cerca de 90% da água encontrada nas carnes (DAMODARAN et al., 2010). Em se tratando da influência da água na qualidade das carnes, sabe-se que aspectos como a distribuição desta na fibra muscular, estado de ligação com as moléculas proteicas e mobilidade no músculo, são tão importantes quanto o total de água presente (RUAN; CHEN, 1998; AURSAND et al., 2008). Neste contexto, a LF ^{1}H NMR é capaz de revelar mudanças nas propriedades do tecido muscular do pescado durante processamento e armazenamento que alterem a mobilidade da água (LAKSHMANAN et al., 2007; GUDJÓNSDÓTTIR et al., 2011; AURSAND et al., 2008).

Tem-se tentado desenvolver métodos que podem ajudar a prever o tempo de armazenamento de produtos de peixe congelado a uma dada temperatura de armazenamento. Tal método, é o método do índice de Qualidade (QIM) (HERRERO et al., 2003) com base na inspeção sensorial. Outras abordagens utilizam técnicas analíticas, por exemplo, Herrero e Careche (2006) construíram um modelo baseado na análise de regressão linear múltipla de combinações de viscosidade aparente de homogeneizados de músculo, punção, e testes de resistência ao cisalhamento para prever o tempo de armazenamento de pescada a -20 ° C. No entanto, estes e outros procedimentos envolvem métodos intensivos e/ou destrutiva de trabalho. Seria altamente desejável desenvolver um método mais rápido, com o potencial de utilização em linha, requerendo a manipulação da amostra mínima.

A LF ^{1}H NMR é um técnica, que como muitas técnicas espectroscópicas, é não destrutiva e não invasiva, exigindo apenas um mínimo de preparação da amostra ou não, e oferece a possibilidade de produzir impressões digitais característicos para uma dada amostra. Além disso, sabe-se que a estrutura e a mobilidade da água são parâmetros importantes, que variam durante armazenamento, e os tempos de relaxação transversal (T_2) medidos por LF ^{1}H NMR foram utilizados para detectar a presença de várias populações de água no tecido muscular (FINCH et al., 1971). Assim, a LF ^{1}H NMR, que vem sendo utilizada para detectar mudanças nos tempos de relaxação das moléculas de água, aparentemente relacionados com as condições de congelamento do bacalhau (JEPSEN et al., de 1999; LAMBELET et al., 1995).

3 DESENVOLVIMENTO

3.1 ARTIGO 1: Classification of Brazilian honeys by physical and chemical analytical methods and Low Field Nuclear Magnetic Resonance (LF ^{1}H NMR). Publisher in LWT - Food Science and Technology (Paper I)

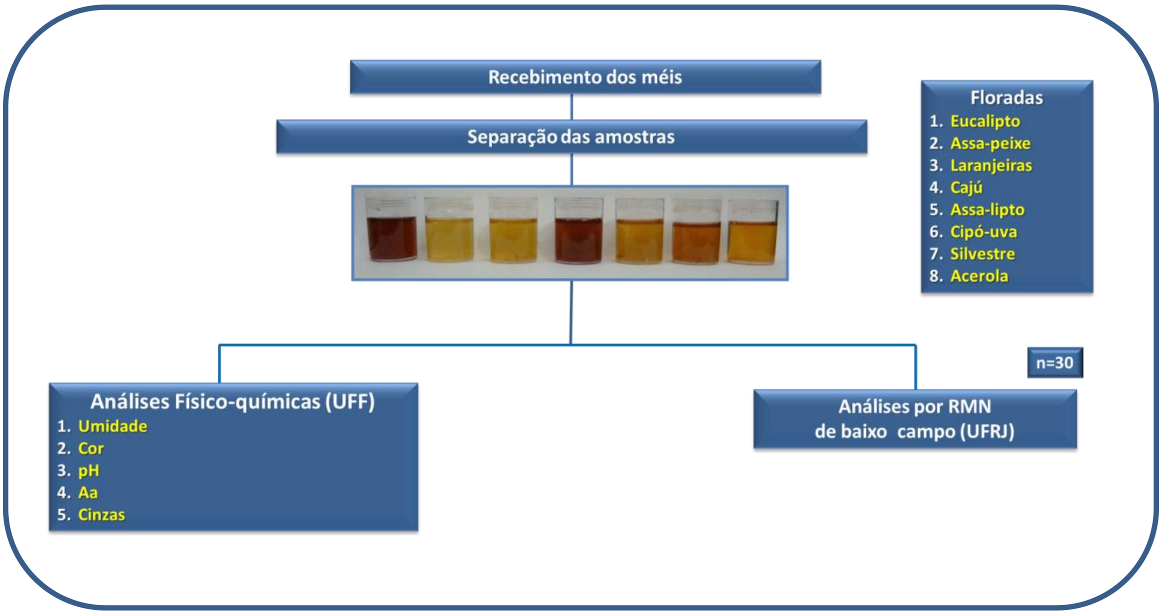

Figura 1. Desenho experimental delineado para desenvolvimento do artigo 1.

Classification of Brazilian honeys by physical and chemical analytical methods and Low Field Nuclear Magnetic Resonance (LF ^{1}H NMR)

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ABSTRACT

This study evaluated the possibility of differentiating the botanical origin of honeys using Low Field Nuclear Magnetic Resonance (LF ^{1}H NMR). Eighty samples of honey from 8 different botanical sources (eucalyptus, “assa-lipto”, oranges, Barbados cherry, cashew tree, “assa-peixe”, “cipó-uva” and polyfloral) were analyzed. A close correlation ($p<0.01$) was established between the LF ^{1}H NMR analysis and physical and chemical measurements, including water content, water activity, pH and color. Bi-exponential fitting of the transverse relaxation (T_2) data revealed two water populations in all samples, T_{21} and T_{22}, corresponding to relaxation times of 0.6 - 1.8 ms and 2.3-5.4 ms respectively. The observed differences in the relaxation times suggest that these were influenced by the differences in botanical origins. Good linear correlations were observed between the T_2 and T_{21} parameters and the physical and chemical data. This study demonstrated that LF ^{1}H NMR can be a viable technique for use in classifying honeys by their botanical origin.

KEYWORDS: Low Field Nuclear Magnetic Resonance (LF ^{1}H NMR); Transverse relaxation; honey; botanical origin; classification.

1. Introduction

Honey composition is influenced by the plant species, climate, environmental conditions, and the contribution of the beekeeper (Anklam, 1998, Azeredo et al., 2003). In general, monofloral honeys are more highly valued than multifloral ones (Andrade et al., 1999). Monofloral honey is produced from nectar that is either wholly or mainly from a single species of plant. These honey types, from defined botanical sources, possess distinctive organoleptic characteristics (Bianchi et al., 2005), and often bring higher prices than honeys from mixed botanical sources.

Many parameters, including pH, ash content, color, optical rotation, proline content, saccharide analysis, and melissopalynological analysis have been proposed by other investigators to classify honeys (Saxena et al., 2010; Terrab et al., 2004; Kahraman et al., 2010; Consonni et al., 2013). In recent decades, quality-control methods, in conjunction with multivariate statistical analysis, have been used successfully to classify honey from different geographical regions, detect adulteration, and describe their chemical characteristics (Cordella et al., 2003; Serrano et al., 2004; Puscas et al., 2013). Also, microscopic (Dimou et al., 2006) and physical and chemical characteristics (Corbella & Cozzolino, 2006; Lazarevic et al., 2012) have been employed for the botanical and geographical determination of honey samples.

Among the analytical methods used to characterize food, in recent decades NMR has achieved general acceptance as a powerful method because of its noninvasive characteristics, high reproducibility, and sensitivity, as demonstrated in a wide range of applications (Karoui et al., 2007, Alam and Alam, 2004). Low Field (LF) ^{1}H Nuclear Magnetic Resonance (^{1}H NMR) is a rapid method for general application, to investigate water mobility in materials and foods (Ruan & Chen, 2001; Martinez et al., 2003; Gudjónsdóttir et al., 2011; Carneiro et al., 2013).

LF ^{1}H NMR can measure proton relaxation and thus can be used to investigate changes in water mobility on food (Andersen & Rinnan, 2002; Aursand et al., 2009; Belton, 1990; Bertram & Andersen, 2007; Bertram, Meyer, & Andersen, 2009). Honey is a very complex multi-component system and its LF ^{1}H NMR relaxation profile can be modeled as a linear combination of characteristic relaxation times from the measurable hydrogens present in their structure. In LF ^{1}H NMR studies, proton relaxation is described by the relaxation time constants T_1 (longitudinal) and T_2 (transverse), where T_2 relaxation decay in food is multiexponential, indicating the presence of different water populations or water “pools” in the foods matrixes (Belton, 1990; Bertram et al., 2001; Cope, 1969; Finch, Harmon, & Muller, 1971). However, we are aware of no published reports on the use of Low Field Nuclear Magnetic Resonance (LF ^{1}H NMR) to classify honey samples of different floral origins.

The aim of this research was to investigate changes in the distribution of water, by means of LF ^{1}H NMR relaxation time measurements associated with physic and chemical measurements of pH, water activity, color, and water, sugar, and ash contents that might enable the classify honey samples of different floral origins.

2. Materials and methods

2.1. Sample

Eighty samples of honey were obtained from beekeepers in different mountain areas in the states of Rio de Janeiro and Minas Gerais in southeastern Brazil. The samples consisted of 10 honeys from each of the following genera and species of plants: eucalyptus (*Eucalyptus* sp.), orange (*Citrus* sp.), Barbados cherry (*Malphigia* sp.), cashew tree (*Anacardium occidentale*), “assa-peixe” (*Vernonia* sp.), “assa-lipto”, and “Cipó-Uva” (*Cissus rhombifolia*), and 10 polyfloral honeys.

All the honey samples collected were stored at room temperature (18-23ºC) from the time of acquisition to the spectral analysis (maximum 4 weeks after the beekeepers removed the honey from the hives). The honey samples were stored in the dark in screw-cap jars.

2.2. Physical and chemical measurements

All measurements were made in triplicate. Physical and chemical analyses were performed following international recommendations (AOAC, 1995).

Water content was determined with an Abbé-type refractometer. All measurements were performed at 20 °C, after waiting for 6 min to allow the sample to reach equilibrium. The refractive index of the honey sample was correlated using Chataway Charts.

The honey pH was measured with a pH meter (Digimed® Model DM-32, São Paulo, Brazil). The electrode was immersed in a suspension made by mixing 10 g of honey with 75 mL of distilled water.

The intensity of the color was measured with a Minolta type CR-400 colorimeter (Konika Minolta®, Tokyo, Japan) according to the manufacturer’s instructions. The instrument records the values for L***** (lightness—intensity of white color), a***** (redness—intensity of red color), and b***** (yellowness—intensity of yellow color).

The water activity was measured with a Pawkit meter (Decagon Devices, Pullman, WA, USA). This measures the water activity of the sample, based on its equilibrium relative humidity (ERH). Approximately 1 g of each honey sample was used to determine the water activity (a_w).

2.3. Low field NMR measurements

For the NMR measurements, a bench-top NMR analyzer with a working frequency of 13 MHz was used (MARAN DXR 2, Oxford Instruments®, Osney Mead, Oxford, UK). The measurements were performed on 15-g samples at 25±1°C in NMR tubes (50 mm diameter). All samples were analyzed in triplicate. The transverse relaxation time (T2) was measured with a Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence (Carr & Purcell, 1954; Meiboom & Gill, 1958), with 10 scans, 256 points, 100 ms between scans, and 100 μs between pulses of 90° and 180°. The LF-NMR relaxation curve was fitted to a multi-exponential curve with the software RI-WINFIT (version 2.5, Oxford Instruments®). T2 distributions were obtained using the software WinDXP, version 1.8.1.0 from Resonance Instruments®, distributed by Oxford Instruments®

2.4. Statistical analysis

A one-way analysis of variance with repeated measures was used to identify differences among the botanical origins of the honeys, for each relaxation time (T_2 and $T_{21,}$ T_{22}) and physical and chemical parameters. When a significant F was found, additional post-hoc tests with Bonferroni adjustment were performed. Pearson correlations were used to examine the relationship between each physical and chemical (pH, water activity, color, and water, ash, and sugar contents) and transverse relaxation parameter (T_2 and T_{21}, T_{22}) independently of botanical origin. Statistical significance was set at the 0.01 level of confidence. All analyses were performed with a commercially available statistical package (SPSS Inc., Version 17.0, Chicago, Illinois, USA).

3. Results and discussion

3.1. Physical and chemical results

Table 1 shows the means, standard deviations and ranges of the data obtained from the analysis of the different physical and chemical parameters (pH, water activity, color, and water, ash, and sugar contents).

Water content was influenced by the climatic conditions, the season of the year, and the degree of maturity of honeys (White, 1978, Conti, 2000; Finola et al., 2007). All samples had low water content, within the limit (20g/100g) allowed for natural honeys (Table 1). In one study, the water content of honeys of different origins showed varietal differences over a range from 16 to 20g/100g (Kayacier & Karaman, 2008). During storage, higher water content could lead to undesirable honey fermentation caused by the action of osmotolerant yeasts, resulting in the formation of ethanol and carbon dioxide. The ethanol can be further oxidized to acetic acid and water, producing a sour taste (Chirife et al., 2006). The mean water content of the samples ranged from 16.20 g/100g for “cipó-uva” to 20.00 g/100g for the cashew honey. The values obtained in this study for monofloral honeys were similar to those obtained by other investigators for Brazilian honeys (Abadio Finco et al., 2010; Welke et al., 2008) of different origins.

The water and sugar contents of honey are closely correlated, as described by Conti (2000). In this study, the regression coefficient was r=0.87 ($p<0.01$).

The carbohydrate composition depends on several factors including the botanical and geographical origins of the plant from which the honeydew or nectar was collected, and the environment, climate, and processing and storage conditions (Marghitaş et al., 2009; Ouchemoukh et al., 2010). Glucose and fructose are the major constituents of honey. In this study, the percentage of reducing sugars in the samples ranged from 66.2 g/100g to 80.1 g/100g. Our findings were similar to those of Ouchemoukh et al. (2007). The values found for total sugar content in this study ranged from 66.7 to 85.4 g/100g. These levels are close to those obtained by Komatsu et al. (2002), Sodré et al. (2007), Arruda et al. (2005) and Moreti et al. (2009), who found total sugar contents of 67.8 to 88.3 g/100g for Brazilian honey samples of different floral origins.

The mean percentage of apparent sucrose was 3.2 g/100g, with a range of 2.2–3.9 g/100g. The level of sucrose differs according to the degree of maturity and origin of the nectar composing the honey. Cantarelli et al. (2008) reported mean sucrose content in honey samples of 4.05 g/100g.

In general, honey is acidic in nature, irrespective of its geographical origin. The pH of honey can be influenced by the different sources of nectar, soil, or combination of plants for honey composition, by the action of glucose oxidase, the action of bacteria as the honey ripens, and also the amount of minerals present in the honey (Finola et al., 2007). Honey pH values are of great importance during storage, as they influence the texture, stability and shelf-life of honeys (Baroni et al., 2009; Downey et al., 2005; Terrab et al., 2004). Most bacteria grow in a neutral and mildly alkaline environment, while yeasts and molds are capable of growth in an acidic environment (pH = 4.0–4.5) (Conti, 2000). The pH values of our samples ranged from 2.98 for “cipó-uva” to 4.15 for cashew-tree honeys. These values fall within the range usually observed for other natural Brazilian honeys, between 3.10 and 4.05 (Azeredo et al., 2003). The pH value of honey is not directly related to free acidity, because of the buffering action of the various acids and minerals present.

The water activity (a_w) of the honey samples varied from 0.57 to 0.70 (Table 1). Our results are quite similar to those for other honeys, for which the a_w values ranged from 0.53 to 0.67 (Lazaridou et al., 2004). The water activity is an important factor in promoting stability, by preventing or limiting microbial growth. However, the osmotolerant yeasts are able to grow at a minimum water activity of 0.6 (Chirife et al., 2006).

The color characteristics are presented in Table 1 with the values for L***** (lightness—intensity of white color), a***** (redness—intensity of red color), and b***** (yellowness—intensity of yellow color). Honey samples having an L value > 50 are considered lighter, and samples having an L* value ≤50 are dark. Based on this classification, the honey samples studied here were relatively light, with L* values ranging from 43.02 to 81.21. The lighter honeys were predominant (87.50%); only the cashew honeys had L* lower than 50. The a* values ranged from 2.91 to 63.40 and b* values ranged from 25.44 to 43.47. Thus it is apparent that all the darker samples had red and yellow components. The color of a honey is closely related to its chemical composition, and is presumed to result primarily from the presence of pigments (carotenoids, flavonoids, derivatives of tannins and polyphenols), which are also known to have antioxidant properties (Frankel et al., 1998). The color values obtained were within the expected ranges for each of the honeys studied. The results of other studies that used L*, a* and b* to measure color were similar to this study (Soria et al., 2004; Terrab et al., 2003). These showed quite low mean values of lightness L*, between 38 and 41, coinciding with their dark color.

The physical property of honey that best correlates with the total mineral content is color. This result is consistent with Ankalm (1998), who reported that dark honeys have a higher mineral content than pale honeys. A predominance of light colors was also previously reported by Moreti et al. (2006) for the 346 honey samples collected in six states in Brazil.

The wide variability of honey composition is also reflected in the ash content (Baroni et al., 2009). Ash content is one of several parameters that have been associated with the botanical and geographical origins of honey samples. The ash content in honey is generally low, and depends on the nectar composition of the predominant plants in its composition (Al-Khalifa & Al-Arify, 1999). In this study, the ash content of the samples ranged from 0.07 g/100 g for “cipó-uva” to 0.42 g/100 g for the cashew-tree honeys. In comparison, other investigators reported the following ranges for natural honeys: 0.28 g/ 100 g (Kahraman et al., 2010), 0.03–0.21 g/100 g (Khan et al., 2006) and 0.08–0.39 g/100 g (Ahmed et al., 2007). The ash percentage was higher in dark honeys such as cashew tree (Table 1).

3.2. Low Field NMR results

No previous studies of the relaxation component with Low Field Nuclear Magnetic Resonance on honeys of different botanical origins are available.

Having established the physical and chemical properties of honeys from 8 different botanical sources, we measured the NMR proton relaxation of these same samples. Our purpose was to evaluate the possibility of a correlation between these data, which could be used to determine the botanical origin of honey. Different methods were used to analyze the T_2 relaxation data: continuous distributed NMR relaxation curves, biexponential fitting of T_2 relaxation curves, and comparison of NMR and physical and chemical data.

3.2.1. Continuous distributed NMR (T_2) relaxation curves

Comparison of the continuous distributed curves revealed visible differences in the distribution of water mobility among the different botanical sources. The cashew honeys, which had higher water activity, tended to exhibit a slightly broader T_2 distribution than did the others with lower water activity. This is likely due to the differences in nectar composition and water distribution caused by different flowers.

3.2.2. Bi-exponential fitting of T2 relaxation curves

Bi-exponential fitting of the transverse relaxation data resulted in the observation of two water populations in all samples, resulting in a faster relaxation time T_{21} in the range of 0.57 to 2.10 ms, and a slower relaxation time T_{22} in the range of 2.30–5.67 ms, depending on the botanical origin (Table 2).

In the chemometric analysis of the data, an ANOVA was carried out to ascertain those variables with mean values that were statistically different for each kind of honey, with a probability of 99% (Table 2). The results for the ANOVA carried out for relaxation time T_{21} showed that the mean contents of all honeys, with the exception of eucalyptus and “assa-lipto”, were significantly different. This indicated that the relaxation time T_{21} of honeys varies according to the botanical origin. On the other hand, the relaxation times T_{22} of all honey samples were statistically different (p<0.01), which confirms the effectiveness of screening of honeys from different botanical origins. This model would allow us to save time in the determination of the floral origin of honey.

3.2.3. Comparison of NMR and physical and chemical data

The NMR data obtained by biexponential fitting were correlated with the physical and chemical parameters. Significant correlations were observed between the NMR relaxation parameters and the physical and chemical properties, with the exception of sugar content (rT_{21}=-0.66; rT_{22}=-0.57). The relaxation times T_{21} and T_{22} showed direct correlations with the water content (rT_{21}=0.92; rT_{22}=0.77), pH (rT_{21}=0.86; rT_{22}=0.73) and water activity (rT_{21}=0.91; rT_{22}=0.83).

In this study, the water activity and pH increases were associated with longer T_{21} relaxation times in honey. We attributed this to the composition of honeys from different botanical origins.

As seen in Fig. 1, water activity was correlated with T_{21} (r = 0.91) for all honey samples. According to Pelizer et al. (2003), the water activity influences microbial growth and enzymatic and biological processes. Each organism has a minimum water activity required to perform its metabolic activities. The optimum water activity for fungi is around 0.70, for yeast 0.80, and for bacteria 0.90.

Values of pH are dependent on the amount of ions in the honey, which is correlated with the total mineral content. Similar correlations were found by Vanhanen et al. (2011), between the total ash content of honey and the pH (r = 0.86). Bertram et al. (2001) also demonstrated that the relaxation times were efficient parameters for indicating changes in muscle pH. Although the change occurred in another matrix, this change in pH demonstrates the direct influences on the relaxation time.

The relaxation times (T_{21}) were correlated with the parameters L* (r=-0.91), a* (r=-0.43), and b* (r=-0.31). The progressively lighter colors in the honeys and the increases in the relaxation time T_{21} were closely correlated with the decrease of the ash content (r=0.87). The mineral content influences the color and taste of honey. The higher the content of metals and the darker the color, the stronger the honey will taste (Sancho et al., 1991). González-Miret et al. (2005) demonstrated that the color of dark honeys (avocado, chestnut, honeydew, and heather) was closely correlated with the concentration of trace elements such as As, Cd, Fe, S, Pb and Ca. On the other hand, the colors of light and brown honeys (citrus, rosemary, lavender, thyme, and eucalyptus) were highly correlated with only Al and Mg.

We also observed a significant correlation between T_{21} and water (r=0.94) content in honeys from different plants. Water content influences honey color, viscosity, flavor, density and refractive index, and is one of the most important physical and chemical parameters for the analysis of conservation and stability of foods in general (Mateo and Bosch-Reig, 1997; Sancho et al., 1991). Estupinan and Sanjuan (1998) reported water contents of honey in the range of 13–25%, and stated that water content was affected by climate, season, and the water content of the original plant nectar.

4. Conclusions

The observations reported here open the way to the development of a tool for classification of honeys. Low Field ^{1}H Nuclear Magnetic Resonance (LF ^{1}H NMR) relaxation is a rapid noninvasive method to investigate the botanical origin of honey. The current reference methods are costly, require sophisticated technical training, and have a long acquisition time. Measurements made through LF^{1}H NMR with appropriate statistical analysis can be extended to accumulate a set of data for classification of the different botanical origins of honey samples, based on reliable clues.

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Table 1. Range of the physicochemical parameters (moisture, pH, ash, Aw and color) of analyzed honeys from different origins botanicals.

Types of Honeys	Moisture [g/100g]	pH	Ash contents [g/100g]	Aw	Color		
					L*	a*	b*
Cashew tree	19.76 ± 0.12^{a} (19.60-20.00)	4.13 ± 0.02^{a} (4.10-4.13)	0.42 ± 0.03^{a} (0.39-0.43)	0.67 ± 0.01^{a} (0.66-0.68)	43.51 ± 0.37^{a} (43.02-43.90)	27.16 ± 0.71^{a} (26.33-28.03)	36.30 ± 0.71^{a} (35.31-36.97)
Polyfloral	19.52 ± 0.13^{b} (19.40-19.80)	4.02 ± 0.03^{b} (4.02-4.10)	0.30 ± 0.02^{b} (0.28-0.31)	0.64 ± 0.02^{b} (0.62-0.65)	53.72 ± 0.44^{b} (53.32-54.33)	3.14 ± 0.16^{b} (2.91-3.26)	26.83 ± 0.98^{b} (25.44-27.52)
Barbados cherry	18.46 ± 0.16^{c} (18.20-18.60)	4.0 ± 0.02^{c} (3.96-4.05)	0.26 ± 0.01^{c} (0.25-0.27)	0.61 ± 0.01^{c} (0.60-0.62)	63.72 ± 0.44^{c} (63.32-64.33)	6.99 ± 0.44^{c} (6.51-7.58)	39.81 ± 0.71^{c} (38.59-40.96)
“Assa-Peixe”	18.28 ± 0.10^{d} (18.20-18.40)	3.94 ± 0.01^{d} (3.93-3.96)	0.19 ± 0.02^{d} (0.17-0.20)	0.60 ± 0.02^{d} (0.59-0.61)	66.07 ± 1.1^{d} (65.65-66.43)	10.44± 0.49^{d} (10.03-11.13)	43.19 ± 0.33^{d} (42.73-43.47)
“Assa-Lipto”	17.75 ± 0.14^{e} (17.60-18.00)	3.92 ± 0.02^{e} (3.90-3.94)	0.18 ± 0.01^{d} (0.17-0.19)	0.58 ± 0.1^{e} (0.57-0.58)	68.65 ± 1.02^{e} (67.24-69.63)	10.44± 0.18^{d} (10.28-10.69)	40.65 ± 0.29^{e} (40.28-40.99)
Eucalyptus	17.29 ± 0.10^{f} (17.20-17.40)	3.79 ± 0.02^{f} (3.78-3.81)	0.12 ± 0.03^{e} (0.39-0.43)	0.57 ± 0.3^{f} (0.55-0.59)	70.55 ± 0.29^{f} (70.14-70.78)	16.39 ± 0.3^{e} (15.45-16.95)	33.08 ± 0.13^{d} (32.93-33.25)
Oranges	16.44 ± 0.20^{g} (16.20-16.40)	3.40 ± 0.01^{g} (3.39-3.42)	0.09 ± 0.02^{f} (0.07-0.10)	0.55 ± 0.01^{g} (0.54-0.56)	75.22 ± 0.32^{g} (74.78-75.49)	11.06 ± 1.14^{f} (9.45-11.95)	40.31 ± 1.51^{ce} (38.23-48.78)
“Cipó-Uva”	16.49 ± 0.1^{g} (16.40-16.60)	2.99 ± 0.02^{h} (2.97.-3.02)	0.07 ± 0.01^{f} (0.06-0.07)	0.53 ± 0.02^{h} (0.51-0.54)	80.79 ± 0.57^{h} (79.98-81.21)	62.57 ± 0.62^{g} (61.90-63.40)	37.19 ± 0.25^{f} (36.87-37.47)

a, b, c . . . Different letters in a column indicate significant differences (*p*<0.01) between treatments (ANOVA).
*L** (darkness), *a** (redness), and *b** (yellowness)
Aw (water activity)

Table 2. Range of the LF ^{1}H NMR parameters obtained in honeys according to the different botanical origins

Types of Honeys	T_{21} [ms]	T_{22} [ms]
Cashew tree	1.88 ± 0.10^a (1.65-2.06)	5.49 ± 0.12^a (5.15-5.68)
Polyfloral	1.58 ± 0.07^b (1.44-1.71)	4.91 ± 0.08^b (4.71-5.05)
Barbados cherry	1.36 ± 0.12^c (1.11-1.63)	3.53 ± 0.10^c (3.33-3.72)
“Assa-Peixe”	1.31 ± 0.07^c (1.16-1.42)	4.07 ± 0.10^c (3.89-4.24)
“Assa-Lipto”	1.07 ± 0.07^d (0.96-1.22)	3.43 ± 0.09^c (3.32-3.59)
Eucalyptus	1.09 ± 0.12^d (0.89-1.32)	2.95 ± 0.09^d (2.75-3.10)
Oranges	0.84 ± 0.05^e (0.73-0.94)	3.30 ± 0.12^c (3.07-3.50)
“Cipó-Uva”	0.64 ± 0.04^f (0.57-0.74)	2.43 ± 0.06^e (2.29-2.54)

a, b, c . . . Different letters in a column indicate significant differences ($p<0.01$) within each treatment (ANOVA).

Figure captions

Figure 1

Fig.1. Correlation between Transverse relaxation time (T_{21}) and Water activity (A_w) in honeys, according to the different botanicals origins.

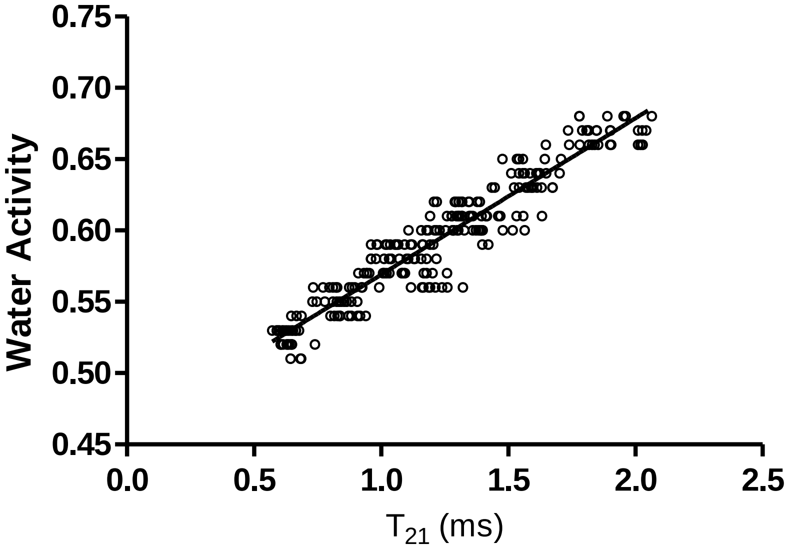

Fig.1. Correlation between Transverse relaxation time (T_{21}) and Water activity (A_w) in honeys, according to the different botanicals origins.

3.2 ARTIGO 2: Detection of honey adulteration by High Fructose Corn Syrup from Low field Nuclear Magnetic Resonance (LF ^{1}H NMR). Under review in Journal of Food Engineering (Paper II)

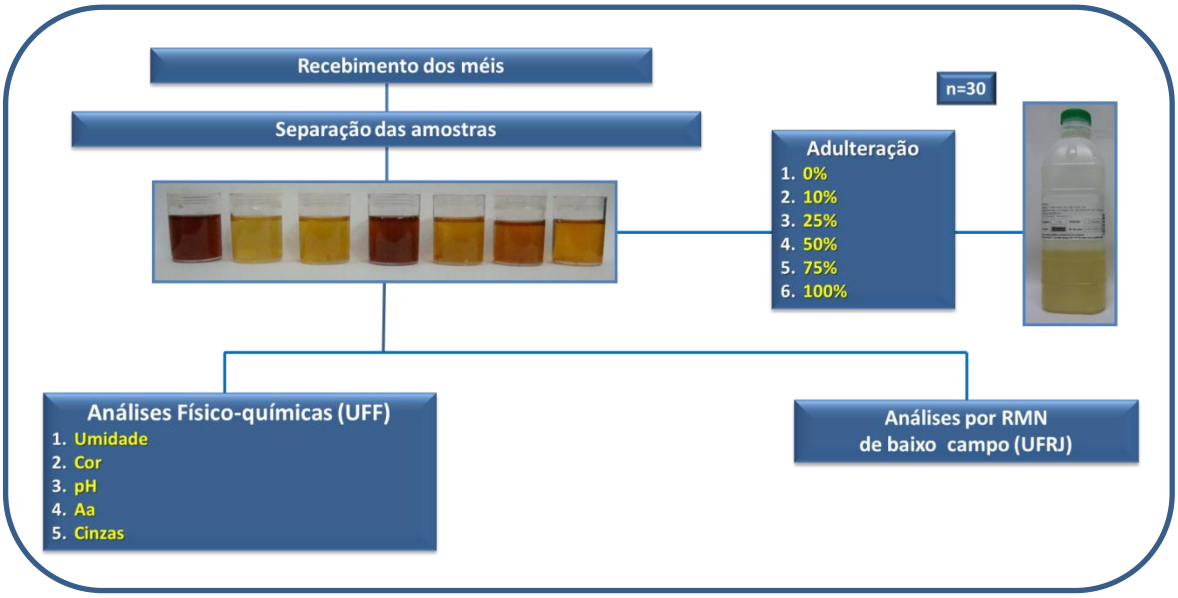

Figura 2. Desenho experimental delineado para desenvolvimento do artigo 2.

Detection of honey adulteration by High Fructose Corn Syrup from Low field Nuclear Magnetic Resonance (LF ^{1}H NMR)

ABSTRACT

The effect of honey adulteration by high fructose corn syrup in different concentrations from 0% (pure honey) to 100% (pure high fructose corn syrup) was investigated using Low-Field Nuclear Magnetic Resonance spectroscopy (LF ^{1}H NMR) and physicochemical analytical methods. The NMR data were analyzed by bi-exponential fitting and compared with physicochemical data. The physicochemical parameters demonstrated that water content, water activity, pH and color differed significantly in honey samples adulterated with different concentrations of high fructose corn syrup. These differences were also observed by transverse relaxation (T_2). Bi-exponential fitting of T_2 resulted in the observation of two water populations in all samples, T_{21} and T_{22}, with relaxation times in the range of 1.26-1.60 ms and 3.33-7.38 ms, respectively. Relaxation times increased with higher percentages of high fructose syrup in adulterated honey. Linear correlations were observed between the T_2, T_{21} and T_{22} parameters and physicochemical data, suggesting that LF ^{1}H NMR can be used to discriminate pure blossom honey from honey adulterated with high fructose corn syrup.

KEYWORDS: Low Field Nuclear Magnetic Resonance (LF ^{1}H NMR); transversal relaxation; adulterated honey; high fructose corn syrups

1. Introduction

The Brazilian annual nectar honey production was estimated at about 40,000 tons for the year 2010 (latest available data). The increase of honey production, which doubled from 18,000 tons in 2000 to 40,000 tons in 2010, ranks Brazil at the 11th position of world-wide producers and has made the country the fifth largest exporter (IBGE, 2010).

Honey has become the target of adulteration with cheaper sweeteners in Brazil as well as in other countries. Honey is subjected to adulteration with inexpensive sweeteners, such as refined cane sugar, beet sugar, high fructose corn syrup and maltose syrup, resulting in higher commercial profits. Consequently, discrimination between adulterated and unadulterated honey and authenticity of honey has become a very important issue for processors, retailers and consumers as well as regulatory authorities. Research for newer, simpler, more sensitive and more economical procedures is ongoing (Ruiz-Matute et al., 2010).

Adulteration of honey can be detected by different analytical techniques, such as near infrared spectroscopy (Zhu et al., 2010), Elemental Analysis - Isotope Ratio Mass Spectrometry (EAIRMS) (Simsek et al., 2012), Fourier transform infrared (FTIR) spectroscopy (Guo et al., 2011), chromatographic (Cordella et al., 2003, Cordella et al., 2005; Moralesa et al., 2008), and nuclear magnetic resonance (Cotte et al., 2007). Although these methods are useful to assess the adulteration of honey, they are time-consuming, destructive and can be expensive, requiring the development of fast, nondestructive, easy-to-use and sensitive analytical methods.

In recent years, LF ^{1}H NMR has gained wide acceptance in the field of food sciences as a powerful method because of its advantages over other analytical techniques. It is a rapid, non-destructive, highly reproducible, and sensitive technique. In combination with chemometric techniques, LF ^{1}H NMR methods are successfully applied in quality control of food products such as porcine muscle (Bertram et al., 2001, Bertram et al., 2007, Straadt et al., 2007 and Bertram et al., 2011), processed pork (Hullberg and Bertram, 2005), salmon (Aursand et al., 2009 and Aursand et al., 2010), shrimp (Gudjónsdóttir et al., 2011, Carneiro et al., 2013), crude oil (Ramos et al., 2009), egg (Laghi et al., 2005), ice cream (Lucas et al., 2005), herbs (Preto et al., 2013), cod (Erikson et al., 2004 and Aursand et al., 2008) and acidified milk drinks (Salomonsen et al, 2007).

The aim of this study was to evaluate the potential of LF ^{1}H NMR parameters and physical and chemical measurements (pH, water activity, color, moisture and ash contents) to differentiate between adulterated (high fructose corn syrup added) and unadulterated pure blossom honey samples.

2. Materials and methods

2.1. Collection and storage of samples

This study was carried out at a farm in the mountainous region of Rio de Janeiro in the South-East of Brazil. Bee colonies were kept in Teresópolis (22º 24' 44" S, 42º 57' 56" W). Pure blossom honey samples (n=30) were collected by beekeepers and stored at room temperature (18-23ºC) from the time of collection to spectral analysis (a maximum of 2 weeks after extraction from the hives). Honey samples were stored in the dark in screw-cap jars at moderate temperatures, to prevent significant changes during storage.

2.2. Sample preparation

Mixtures were prepared using pure blossom honey samples and high fructose syrup. Pure model mixtures were prepared by adding high fructose syrup to honey samples at a ratio of 0%, 10%, 25%, 50%, 75% and 100% by weight. After adding the high fructose syrup, samples were kept in a water bath at 35ºC for 20 min and stirred for 1 min followed by cooling in an ice bath.

2.3. Physicochemical measurements

Moisture was determined with an Abbe refractometer. All samples were measured at 20ºC after a 6 minute wait time to allow them to reach temperature equilibrium with the refractometer (AOAC, 1990). The refractive index of honey samples was correlated using Chataway Charts.

The pH of honey was measured with a pH meter (Digimed® Model DM-32, São Paulo, Brazil). The electrode was immersed in a suspension made by mixing 10 g of honey with 75 mL of distilled water (AOAC, 1990).

Color intensity was measured with a Minolta CR-400 colorimeter (Konika Minolta®, Tokyo, Japan) according to the manufacturer's instructions. The instrument records *L*** (lightness—intensity of white color), *a*** (redness—intensity of red color) and *b*** (yellowness—intensity of yellow color) values.

Ash contents were determined with a weighed sample ignited in a muffle furnace at 550ºC until a constant weight for carbonization was reached.

Water activity (a_w) was measured with a Pawkit meter, by Decagon Devices, USA, using a small aliquot of honey with a weight of approximately 1 g. This measure of water activity is based on the equilibrium relative humidity (ERH) of the sample. Measurements were performed in triplicates for each sample and the mean was determined.

2.4. Low field NMR measurements

For the NMR measurements, a bench-top NMR analyzer with a working frequency of 13 MHz was used (MARAN DXR 2, Oxford Instruments®, Osney Mead, Oxford, UK). Measurements were performed on 15 g samples at 25±1°C in NMR tubes (50 mm diameter). All samples were analyzed in triplicate. The transverse relaxation time (T_2) was measured with a Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence (Carr & Purcell, 1954; Meiboom & Gill, 1958), with 10 scans, 256 points, 100 ms between scans, and 100 µs between pulses of 90° and 180°. The LF-NMR relaxation curve was fitted to a multi-exponential curve with the software RI-WINFIT (version 2.5, Oxford Instruments®). T_2 distributions were obtained using the software WinDXP, version 1.8.1.0 from Resonance Instruments®, distributed by Oxford Instruments®.

2.5. Statistical analysis

A one-way analysis of variance (ANOVA) with repeated measures was used to identify differences between pure and adulterated honey for each relaxation time (T_2 and $T_{21,}$ T_{22}) and physicochemical parameters. When a significant F was found, additional post-hoc tests with Bonferroni adjustment were performed. Pearson correlations were used to examine the relationship between each physicochemical (moisture, pH, water activity, ash content, sugar content and color) and each transversal relaxation parameter (T_2 and T_{21}, T_{22}). Statistical significance was set at the 0.01 confidence level. All analyses were performed with a commercially available statistical package (SPSS Inc., Version 17.0 Chicago, Illinois).

3. Results and discussion

We measured the NMR proton relaxation of honey samples adulterated with high fructose syrup and of unadulterated pure blossom honey samples and established their physical and chemical properties. Our goal was to evaluate the relationship between these parameters, and to test its applicability to the discrimination of pure honey from adulterated honey of different percentages. A variety of methods was used to examine the T_2 relaxation data: continuous distributed NMR relaxation curves, biexponential fitting of T_2 relaxation curves, and comparison of NMR and physical and chemical data.

Comparison of the continuous distributed curves revealed visible differences in the distribution of water mobility among the different percentages of adulterated honeys. The 100% adulterated samples tended to show a slightly broader T_2 distribution than other treatments. This behavior can be explained by high water activity in fructose syrup. T_2 has been related to water activity in other matrixes (Bertram et al., 2007, Aursand et al., 2010, Carneiro et al., 2013). These differences can be explained by the amount of syrup added, which affects the water activity in adulterated samples.

The LF ^{1}H NMR relaxation time method was used to investigate water mobility in honey samples. By bi-exponential fitting of the transverse relaxation data we were able to discriminate two water populations in all samples, one with a faster relaxation time T_{21} in the range of 1.26 to 1.60 ms, and another with a slower relaxation time T_{22} in the range of 3.33 to 7.38 ms, depending on the percentage of adulteration with high fructose syrup (Table 1). T_{21} (faster relaxation time) was slower in pure blossom honey (1.26-1.51 ms), indicating that water mobility was lower in unadulterated honey compared to the adulterated honey.

We detected significant differences of moisture, pH, water activity, ash content and color ($p<0.01$) between adulterated and pure blossom honey (Table 2). ANOVA results (Table 2) showed that at relaxation time T_{21} all adulterated honey samples, including those at the 10% level, differed significantly from pure honey. This indicates that the relaxation time T_{21} changes in honey adulterated with high fructose syrup. Additionally, relaxation times T_{22} of all honey samples differed significantly from each other ($p<0.01$), allowing to discriminate different percentages of adulteration. This model thus confirms the effectiveness of the screening and suggests that it can be a time-saving method for detecting high fructose syrup adulteration of honey.

Moisture, water activity and pH increase were associated with longer T_{21} and T_{22} relaxation times in honey and were related to the percentage of adulteration with high fructose syrup. Knowledge of the moisture in honey is useful to improve conservation and storage. High levels of humidity are important for honey because they affect other characteristics such as viscosity, fluidity and conservation (Acquarone et al., 2007). The moisture content of pure honey (17.65%) increased gradually as the percentage of the adulterant was raised (Table 2). As shown in Figure 1, we observed a significant negative correlation between T_{22} and moisture (r=-0.92). At the 50% adulteration level the moisture content of 20.2% was close to the allowed limit for natural honeys (20%), which represents the critical moisture level for the keeping quality of honey (Codex Alimentarius, 2001 and EU Council, 2002). In contrast, at the 75% and 100% adulteration level, moisture exceeded the recommendation by the legislation.

Water activity was negatively correlated with T_{21} and T_{22} (r = -0.86 and r= -0.91, respectively). This parameter is important for stability, preventing or limiting microbial growth (Pelizer et al. 2003). Furthermore, other differences in the experimental conditions such as high fructose syrups content can affect the relaxation times (T_2 and T_{21}, T_{22}).

The pH values ranged from 3.10 to 4.70 for pure honey and adulterated honey samples. This parameter is of great importance during extraction and storage of honey because it influences its texture, stability and shelf life (Gomes et al., 2011). The low pH of honey inhibits the presence and growth of microorganisms and causes the honey to be compatible with many food products in terms of pH. A raise the percentage of the adulterant resulted in significantly increased pH values, and was also reflected in longer T_{21} and T_{22} relaxation times of adulterated honeys.

Ash values depend on the mineral content of honey and directly measure inorganic residue after carbonization. In this study, the ash content of pure blossom honey (0.15%) increased gradually as the percentage of adulterant was increased. Ash content was highly negatively correlated with relaxation time T_{22} (r = -0.90).

The color of the analyzed samples ranged from yellow to brown, depending on the percentage of adulteration. The color characteristics are summarized in Table 2 (means, standard deviations and ranges of the parameters *L**, *a** and *b**). Relaxation times (T_{22}) were correlated with the parameters *L** (r = -0.87), *a** (r = -0.52), and *b** (r = -0.29). The plot of parameters a* and b* shows that the analysed honey samples had red, yellow and green components. Green components were indicated by the presence of negative a* values in all samples, although *a** and *b** did not significantly differ in adulterated honeys.

The highest correlation coefficient found was for the relationship between T_{22} relaxation time and the *L** value of honey color (r = –0.87). The correlation was negative, indicating that lighter honeys have higher *L** values due to the percentage of adulteration. The progressively lighter colors and an increase in the relaxation time T_{22} were closely correlated with the decrease of the ash content (r = 0.84). The mineral content influences the color (González-Miret et al., 2005). The higher the content of metals and the darker the color, the stronger the honey will taste (Sancho et al., 1991). Surprisingly, the color of honeys in this study was also noticeably different and varied from pale yellow to dark brown.

4. Conclusions

In the present study, LF ^{1}H NMR was used to discriminate pure blossom honey from honey adulterated with high fructose corn syrup. Our results indicate that adulterated honey samples can be satisfactorily discriminated from pure blossom honey by using LF ^{1}H NMR. Relaxation times were significantly affected by adulterate concentration in pure honey, decreasing at higher fructose syrup concentrations. Significant correlations were found between relaxation times and physical-chemical parameters (pH, water activity and moisture content). We therefore conclude that LF ^{1}H NMR can be used to discriminate pure blossom honey from honey adulterated with high fructose syrup. However, in order to improve the model accuracy and stability, further studies need to test additional types of honey.

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Table 1. Range of the LF ^{1}H NMR parameters obtained in honeys according to samples adulterated with high fructose corn syrup

Adulteration (%)	**T_2 [ms]**	**T_{21} [ms]**	**T_{22} [ms]**
Pure honey	5.90 ± 0.07^a (5.74-5.94)	1.60 ± 0.08^a (1.45-1.79)	7.38 ± 0.10^a (7.23-7.54)
10	5.30 ± 0.10^b (4.73-5.55)	1.51 ± 0.10^b (1.36-1.67)	6.55 ± 0.28^b (5.90-6.91)
25	5.19 ± 0.11^c (5.05-5.31)	1.57 ± 0.12^b (1.42-1.79)	6.26 ± 0.25^c (6.15-6.53)
50	4.21 ± 0.07^d (4.11-4.34)	1.48 ± 0.10^c (1.41-1.72)	5.69 ± 0.19^d (5.44-6.00)
75	3.26 ± 0.12^e (3.08-3.79)	1.32 ± 0.16^d (1.16-1.36)	4.00 ± 0.18^e (3.78-4.55)
100	2.67 ± 0.6^f (2.61-2.75)	1.26 ± 0.8^d (1.12-1.32)	3.33 ± 0.07^f (3.54-3.87)

a, b, c . . . Different letters in a column indicate significant differences (p<0.01) between treatments (ANOVA).

Table 2. Ranges of physicochemical parameters (moisture, pH, ash, Aw and color) for honey adulterated with different percentages of high fructose corn syrup.

Adulteration (%)	Moisture [g/100g]	pH	Ash [g/100g]	Aw	Color		
					*L**	*a**	*b**
Pure honey	17.60 ± 0.5^{a} (17.40-17.80)	3.10 ± 0.04^{a} (3.08-3.15)	0.15 ± 0.1^{a} (0.13-0.17)	0.51 ± 0.02^{a} (0.50-0.53)	48.17 ± 2.12^{a} (46.86-50.35)	7.88 ± 0.44^{a} (7.61-7.98)	40.22 ± 1.84^{a} (38.59-40.96)
10	18.20 ± 0.8^{b} (18.00-18.60)	3.30 ± 0.03^{b} (3.29-3.42)	0.17 ± 0.2^{b} (0.16-0.18)	0.55 ± 0.3^{b} (0.54-0.57)	51.79 ± 1.57^{b} (49.98-53.21)	3.25± 0.49^{b} (3.03-3.96)	38.19 ± 2.61^{b} (37.52-39.97)
25	19.40 ± 0.10^{c} (19.00-19.60)	3.68 ± 0.05^{c} (3.66-3.69)	0.20 ± 0.1^{c} (0.20-0.21)	0.58 ± 0.08^{c} (0.57-0.60)	55.95 ± 2.29^{c} (53.17-57.68)	8.44± 1.18^{a} (8.28-9.49)	36.65 ± 1.59^{b} (34.28-38.09)
50	20.20 ± 0.7^{d} (20.0-20.60)	3.82 ± 0.02^{d} (3.80-3.84)	0.25 ± 0.3^{d} (0.23-0.26)	0.60 ± 0.01^{c} (0.59-0.60)	68.65 ± 3.02^{d} (67.44-70.81)	10.35 ± 1.3^{c} (9.45-11.25)	33.28 ± 1.43^{c} (32.81-33.45)
75	20.80 ± 0.6^{e} (20.40-21.00)	3.98 ± 0.03^{e} (3.97-4.00)	0.29 ± 0.3^{e} (0.27-0.30)	0.66 ± 0.02^{d} (0.65-0.67)	72.24 ± 1.10^{e} (62.38-63.27)	11.33 ± 1.14^{c} (10.12-13.25)	30.31 ± 1.51^{d} (28.13-31.68)
100	22.80 ± 0.7^{f} (22.60-23.00)	4.70 ± 0.08^{f} (4.65-4.80)	0.31 ± 0.2^{f} (0.30-0.32)	0.71 ± 0.01^{e} (0.70-0.72)	74.60 ± 1.36^{f} (63.46-65.26)	6.57 ± 0.62^{d} (6.20-7.42)	27.74 ± 2.25^{e} (26.87-29.47)

a, b, c . . . Different letters in a column indicate significant differences (p<0.01) between treatments (ANOVA).
*L** (darkness), *a** (redness), and *b** (yellowness)
Aw (water activity)

3.3 ARTIGO 3: Atlantic cod (*Gadus morhua*) evaluated by physicochemical analytical methods and Low Field Nuclear Magnetic Resonance (LF ^{1}H NMR). Submitted to LWT - Food Science and Technology (Paper III)

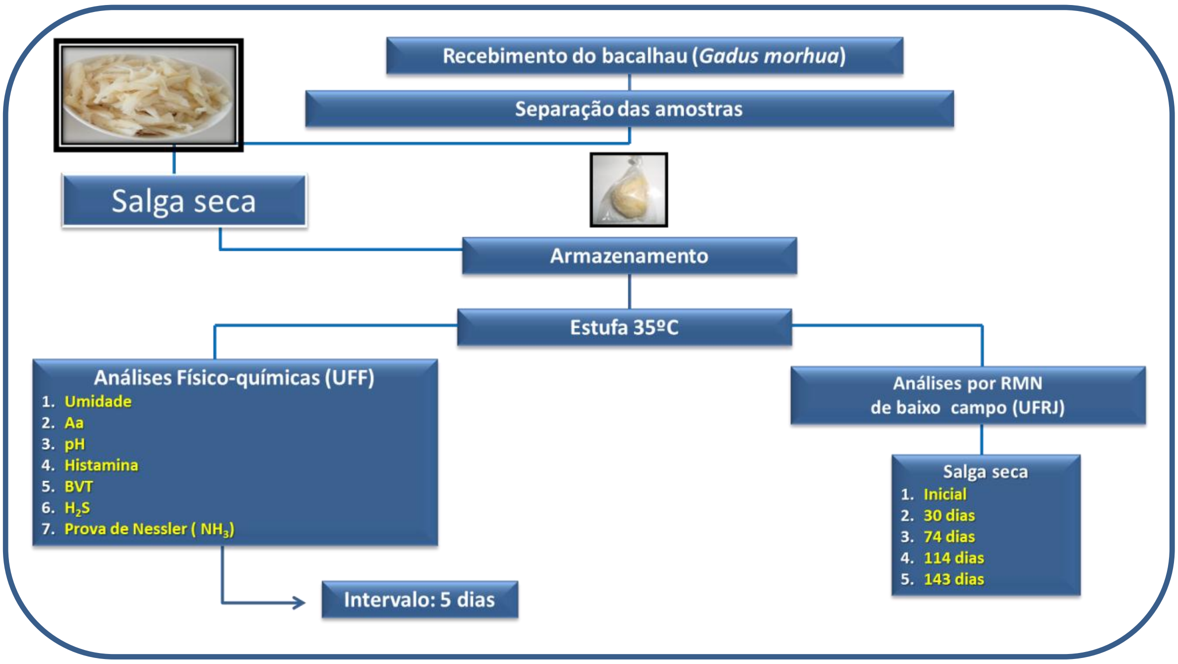

Figura 3. Desenho experimental delineado para desenvolvimento do artigo 3.

Atlantic cod (*Gadus morhua*) evaluated by physicochemical analytical methods and low field nuclear magnetic resonance (LF ^{1}H NMR)

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ABSTRACT

Low Field Nuclear Magnetic Resonance (LF ^{1}H NMR) transverse relaxation was used in comparison to physicochemical parameters to measure water mobility and distribution in Atlantic cod fillets (*Gadus morhua*) during storage. Transverse relaxation time (T_2) identified two water populations with different relaxation times: T_{21} ranging from 6.86 to 14.78 ms, and T_{22} ranging from 45.82 to 65.55 ms. Significant correlations were observed between NMR relaxation parameters and physicochemical quality properties. The relaxation parameters reflected the differences in muscles caused by protein degradation and change in muscle properties during storage. Our study tested the utility of LF ^{1}H NMR as an indicator for the deterioration of cod quality by biochemical mechanisms that affect water properties and muscle structure. The results suggest that LF ^{1}H NMR is a useful tool to evaluate cod quality and can be a good replacement for the time-consuming and sample-destructive traditional physicochemical measurements.

KEYWORDS: Transversal relaxation time; Atlantic cod (*Gadus morhua*); water distribution; water mobility, cod storage.

1. Introduction

Atlantic cod currently is the largest cod stock in the world, and probably has been in the past (Yaragina et al., 2011). It is one of the largest export products from Iceland and Norway with major markets in Southern Europe and Latin America. Salting of foods is a traditional preservation method, where water properties strongly influence food quality. Based on the principle of salting, improving basic knowledge about water mobility in muscle tissue is important (Boudhrioua et al., 2009).

Brine concentration and temperature are the main factors affecting the rates of water and salt diffusion (Bellagha et al., 2007; Boudhrioua et al., 2009; Poernomo et al., 1992). Conservation by salting leads to the diffusion of water and swelling of muscle fibers (Offer and Knight, 1988 and Offer and Trinick, 1983). The uptake of salt in fish muscle tissue affects muscle properties, such as water holding capacity (WHC), which increases with rising salt concentrations of up to 6% (Gudjonsdottir et al., 2010). Water is one of the most important components for the quality of food matrices including fish muscle. Water influences quality attributes such as appearance, texture and storage stability. The water content of fish muscles differs between populations depending on how tightly the water molecules are bound to the muscle structure (Huff-Lonergan and Lonergan, 2005). Water state and mobility, as well as the total amount of water are important for the overall quality of the product (Ruan and Chen, 2001). Understanding the effects of processing and transport on shelf life and product quality is a prerequisite for optimizing the quality and value of fresh fish products.

The physicochemical and enzymatic changes that occur during storage affect protein structure and thus indirectly affect the mobility of the water contained in food (Ordóñez et al., 2005; Gonçalves et al, 2008). Therefore, there is a need to investigate alternative methods that enhance storage ability (Társico et al. 2006).

Low field nuclear magnetic resonance (LF ^{1}H NMR) can be a good substitute of traditional physicochemical analysis because, like many spectroscopic techniques, it is non-destructive and non-invasive, and requires minimal or no sample preparation by producing characteristic fingerprints for a given sample (Gudjónsdóttir et al., 2011). LF ^{1}H NMR can measure proton relaxation and thus can be used to analyze changes in water mobility in food

(Andersen & Rinnan, 2002; Aursand et al., 2009; Bertram & Andersen, 2007; Carneiro et al., 2013; Ribeiro et al., 2014). Moreover, water structure and water mobility are relevant parameters that vary during technological procedures and the transversal relaxation times (T_2) measured by LF NMR indicate the presence of several water populations in muscle tissue (Gudjónsdóttir et al., 2011). Thus, LF ^{1}H NMR, which has already been used to detect changes in the relaxation times of water molecules apparently related to frozen storage conditions in cod (Andersen & Rinnan, 2002; Lambelet et al., 1995) may be a suitable technique to predict quality changes in muscle products. Multi-way chemometric analysis of LF NMR relaxation values allowed the identification of several water populations (Jensen et al., 2005).

Our aim was to follow the biochemistry changes in Atlantic cod muscle that occur during storage by means of LF-NMR relaxation time measurements. The LF ^{1}H NMR parameters obtained were compared to physicochemical measurements of moisture contents, water holding capacity (WHC), water activity (a_w) and pH.

2. Materials and methods

2.1. Sample

Seven kilograms of Atlantic Cod (*Gadus morhua*) loin were obtained in 2012. Individual fishes were cut into small pieces with a stainless steel knife and pooled. For LF ^{1}H NMR analysis, 300 samples of 13g each were prepared. The samples were separated into 30 groups of 10 samples with numbered plastic tags for identification. 30 plastic bags, each containing 60 grams, were separated for physicochemical measurements. All samples were stored in controlled atmosphere at an abusive temperature (38±2°C) for 114 days.

Physicochemical analyses were performed weekly. LF ^{1}H NMR was performed after 0, 30, 74 and 114 days of storage when the quality analysis detected chemical alterations.

2.2. Physicochemical measurements

The moisture analyses were performed with an Infrared Dryer (ID) (Mettler-Toledo GmbH® Model LJ16, Greifensee, Switzerland), a compact instrument with an integrated balance and an electromagnetic radiation lamp. This method of drying involves the heating of samples. Measurements were performed with 5.0 ± 0.1 g of triturated cod muscle (Carneiro et al., 2013).

The pH was measured with a pH electrode connected to a pH meter (Digimed® Model DM-32, São Paulo, Brazil). The electrode was immersed in a suspension made by mixing 10 g of minced cod with 60 mL of distilled water (Conte-Junior et al., 2008).

The water holding capacity (WHC) of cod muscle was adapted by Akse et al. (1993) and Lakshmanana et al. (2007). An aliquot (2.0 ± 0.1 g, four digits) was weighed and placed in a pre-weighed tube with a pre-weighed piece of filter paper. Samples were centrifuged at 210g for 5 min at 4ºC (Hermle® Z 360K Centrifuge, Germany). The loss of water by centrifugation was calculated as the difference in weight before and after centrifugation. The water holding capacity (expressed as % WHC) was calculated as the ratio of remaining water content to pre-centrifugation water content.

2.3. LF ^{1}H NMR Sample Preparation and Analyses

LF ^{1}H NMR sample preparation and analyses were performed according to Carneiro et al. (2013). For the NMR measurements, a bench-top NMR analyzer with a working frequency of 13 MHz was used (MARAN DXR 2, Oxford Instruments®, Osney Mead, Oxford, United Kingdom). The measurements were performed on 13g samples at 25±1ºC in NMR tubes (50 mm diameter). The transverse relaxation time (T_2) was measured with a Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence (Carr & Purcell, 1954; Meiboom & Gill, 1958), with 12 scans, 2048 points, 3.5 s between scans, and 300 ms between pulses of 90º and 180º. The LF-NMR relaxation curve was fitted to a multi-exponential curve with the software RI-WINFIT (version 2.5, Oxford instruments®). Analysis of the exponential fits indicated that two exponentials were sufficient to describe the system for all samples. Bi-exponential fitting thus resulted in two water populations, A_{21} and A_{22}, with corresponding relaxation times T_{21} and T_{22}. T_2 distributions were obtained using the software WinDXP, version 1.8.1.0 from Resonance Instruments®, distributed by Oxford Instruments®.

2.5. Statistical analysis

Means and standard deviations of moisture content, WHC, a_w and pH were calculated and one-factor analysis of variance (ANOVA) was performed for each of these parameters to compare storage time. Data were analyzed using GraphPad Prism®, (GraphPad Software, San Diego, California, USA) at a 95% confidence level. We computed a Principal Component Analysis (PCA) and Partial Least Regression (PLS) analyses, with storage time as Y-variable. Correlations between LF ^1H NMR parameters, and between these and WHC, pH, a_w and moisture were performed using the software XLSTAT version 2012.6.08 (Addinsoft, Paris, France).

3. Results and discussion

Ranges of physicochemical measurements and LF ^1H NMR data obtained during storage are shown in Table 1 and Table 2, respectively. Various methods were used to analyze T_2 relaxation data: continuous distribution of LF ^1H NMR relaxation curves, bi-exponential fitting of T_2 relaxation curves and comparison of LF ^1H NMR and physicochemical data.

3.1. Physicochemical results

Moisture content, water holding capacity (WHC), pH and water activity of Atlantic Cod (*Gadus morhua*) after storage for 114 days are shown in table 1.

On day 1 of measurements, mean pH value was 6.57. At the end of the experiment this value increased to 6.92 ($p<0.05$). These results are not in agreement with other studies where fish muscle pH did not vary throughout storage (Arkoudelosa et al., 2007; Li et al., 2011). The change in mean pH value of 6.64 on day 30 did not differ significantly from previous values. The increase was most prominent after 74 days of storage ($p<0.05$). Gudjónsdóttir et al. (2011) evaluated structural changes in fresh cod muscle occurring with dry salting and rehydration and found a pH of 7.1. These authors explained this by an increase of pH due to salting, an important factor in fish water retention, because it shifts the protein away from its isoelectric point (pH~5.1). This increases the distance between the polypeptide chains and therefore the space for water retention.

The water holding capacity (WHC) is related to the pH of muscles (Rustad et al., 2000). In this study, pH values were inversely proportional to WHC. In accordance with Herrero et al. (2005) and Sánchez-Alonso et al. (2012), WHC decreased with storage time. It dropped from approximately 60 g/100 g from the first day of storage to about 43 g/100 g at the end of the experiment (Table 1). The effect of storage on water retention is correlated with the changes in muscle pH, which alter the water retention capacity of proteins (Herrero et al. (2005). Until day 30 of storage, mean WHC was similar (60.7 and 59.5 g/100 g, respectively ($p>0.05$)). On day 74 and day 114, WHC was lower (49.2 and 43.1 g/100 g) ($p<0.05$). For the duration of the experiment, moisture content increased significantly ($p < 0.05$) with advanced storage time, from 49.6 % to 60.1 %. A significant difference in moisture uptake was found between day 30 and day 114, but not between day 1 and day 30.

Osmotic dehydration is a technique used to reduce a_w to improve nutritional, sensorial and functional properties of food. It is achieved by the immersion of a food product in a concentrated solution of salts and/or low molecular weight carbohydrates (Yao and Le Maguer, 1996). The difference in osmotic pressure between the food and the surrounding solution acts as a driving force for water removal, while the complex cellular structure of the food acts as a semipermeable membrane (Byrne et al., 2001). We observed a significantly higher a_w with advanced storage time (0.74–0.90). This was a general trend on all days of the evaluation.

3.2 Study of water populations by LF ^1H NMR analysis

We measured NMR proton relaxation using various methods to analyze the T_2 relaxation data: continuous distribution of NMR relaxation curves, bi-exponential fitting of T_2 relaxation curves, and comparison of NMR and physicochemical data.

The variation in fish quality during storage time had a pronounced effect on T_2 distributions. A comparison of the continuous distribution curves revealed visible differences in water mobility during storage time. The T_2 distribution on the first day of storage was slightly broader than on other days, which is most likely due to the differences in muscle composition and water distribution caused by storage time.

By bi-exponential fitting of the transverse relaxation data, we detected two water populations. The population with intermediate relaxation time (6.86–14.78 ms), termed T_{21}, is considered to be water associated to, or enclosed in, highly organized structures, such as water bound to tertiary or quaternary protein structures or located in spaces with high density of myofibrils, including actin and myosin filaments. The second population, T_{22}, with a longer relaxation time (45.82–65.55 ms), corresponds to extra-myofibrillar water (Aursand et al., 2008), probably associated with myofibrillar water and water within the protein structure, in all samples during storage (Table 2). This is not in agreement with Aursand et al. (2008) who found T_{21} and T_{22} values for fresh and brined cod to be in a range of 40–60 ms and 150–400 ms, respectively. Relaxation times of samples during storage did not vary in their study.

In our study, both value and amplitude of T_{21} decreased while T_{22} and its amplitude increased with advanced storage time (Table 2). There is a progressive decrease in the water population with a relaxation time close to 12 ms and an increase in the amount of water populations with higher relaxation times (45–65 ms), with widening profiles of the latter.

Changes in the amplitudes of both water populations (A_{21} and A_{22}) were more pronounced during the final days of storage. The changes in A_{21} were more prominent than in A_{22}. There was a high ($0.93 \leq R^2 \leq 0.97$) and significant correlation ($p < 0.05$) between the values of T_{21}, A_{21} and A_{22} estimated by biexponential methods, but not with T_{22} values ($R^2 = 0.33$).

We did not detect free water (with a well-known relaxation time close to 2 s), which presumably corresponds to water less bound than intermyofibrillar or extracellular water. This may explain in part the differences in the evolution between the T_{22} measured by method biexponential fitting would have forced this additional water population to be part of the T_{22} population.

The values of T_{21} and T_{22} as well as their respective populations A_{21} and A_{22}, are within the range observed in frozen cod mince by Sánchez-Alonso (2012), even though we found less variation during the storage period. This can be attributed to the fact that minced muscle deteriorates faster than intact fillet but also to the species studied and to experimental differences.

To analyze the overall variation in the LF ^1H NMR relaxation parameters (T_2 and T_{21}, T_{22}) a principal component analysis (PCA) was performed on the raw transverse relaxation data. The relaxation curves were maximum normalized for taking into account only the water distribution, while ruling out effects of the absolute water content. The first principal component (PC1) explained 99.97% of the variation, mainly describing the effect of storage time on relaxation parameters of Atlantic Cod (T_2, T_{21} and T_{22}). The correlation loadings from the first two PCs are listed in Fig. 1, which shows the PCA results for the normalized transversal relaxation decays of all samples. This increasing dimensionality also helps to discriminate between highly related samples (different days of storage) of complex matrices (relaxation times). The results show the difference between samples during the forced storage time. The T_2 data reflect a significant difference in relaxation time after 74 days of storage. Data are clustered in four separate groups, with greater distances to day 1 as storage time increased (Figure 1).

3.3. Comparison of LF ^{1}H NMR and physicochemical data

LF ^{1}H NMR is a fast and non-destructive analytical method, which in many cases could replace the more time-consuming and often sample-destructive physicochemical methods if strong correlations between the LF ^{1}H NMR parameters and relative physicochemical parameters were found. To analyze the relationship between the LF ^{1}H NMR relaxation parameters and various physicochemical quality factors, such as moisture, a_w, pH and WHC, a weighted principal component analysis (PCA) of the fitted LF ^{1}H NMR relaxation parameters and the physicochemical parameters (Fig. 1) was performed. LF ^{1}H NMR data were compared to established methods to better understand the dynamics of water and how LF ^{1}H NMR can be interpreted with regard to muscle and water behavior.

The PCA bi-plots of the first two PCs describe 99% of the total sample variation. Sample groupings were similar to the NMR T_2 relaxation PCAs in Figure1, but here allowed an overall view of the physicochemical and NMR relaxation characteristics of the samples.

Storage conditions were characterized by high moisture, pH and water activity, coupled with long T_{22} relaxation times, while WHC decrease was coupled with shorter T_{21} relaxation times.

To assess the significant correlation of LF ^{1}H NMR parameters with physicochemical properties ($P < 0.05$), measured by Partial Least Square (PLS1), models were built for each physicochemical parameter as the Y-matrix and each physicochemical quality parameter as the Y-parameter (Figure 2). Significant correlations were found between various LF ^{1}H NMR parameters and all physicochemical quality parameters. However, all LF ^{1}H NMR parameters contributed significantly to the estimation of quality changes of Atlantic Cod during storage. The multivariate PLS1 models showed that experimental design variables significantly affected all NMR parameters when all samples were analyzed together.

When the samples were analyzed in groups, more significant correlations between NMR parameters and physicochemically measured parameters were found, especially for moisture content and WHC. Decreasing WHC was associated with shorter T_{21} relaxation times, indicating decreased restriction of water due to storage, regardless of storage time. This is in agreement with the fact that muscle tissue can wither when samples are stored for a long time, leading to improved WHC as reflected by shorter T_{21} values. Jepsen et al. (1999) showed that the WHC could be predicted using low field NMR within a range of 30–90 g/100g in cod.

T_{21} and T_{22} relaxation times contributed significantly to the prediction of WHC when all samples were analyzed together. In addition, T_2 correlated well with moisture content during storage. In accordance with Herrero et al. (2005), WHC decreased with storage time. Such a decline has been related to changes in water–protein interactions (Offer & Knight, 1988). It dropped from approximately 50 g/100 g after 74 days of storage to about 43 g/100 g at the end of the experiment. This parameter is important for evaluating fish quality. We previously reported changes in WHC of shrimp treated with a higher $Na_5P_3O_{10}$ content (50 g/L) and longer contact time (120 min) (94.8 g/100 g). The results indicate that LF ^{1}H NMR can be used to identify changes in water mobility in shrimp muscle treated with the additive. This assumption was based on the fact that the effect of phosphates on water retention was correlated with the changes in muscle pH caused by the additive, thus altering the water-retention capacity of the proteins (Carneiro et al., 2013).

A strong correlation between muscle pH and relaxation time T_{21} was found (r=-0.93). This is in agreement with the study of Currie, Jordan, & Wolfe (1981) who demonstrated that changes in beef muscle water measured by longitudinal relaxation times T_1 were highly sensitive to changes in pH post mortem. Gudjónsdóttir et al. (2011) also found strong correlations between NMR parameters and ultimate pH in the injected brine and in low-salt samples of cod (*Gadus morhua*), indicating that pH can affect the pore size distribution within the muscle.

Significant correlations were recorded between T_2 values and moisture and a_w ($-0.90 \geq R^2 \geq -0.88$). Interestingly, a significant correlation was found between relaxation times (T_{21}) and moisture and a_w (r=-0.94 and r=-0.92, respectively). Moisture is considered a good indicator for storage quality loss and correlations have been found with T_{22} in stored brine-injected cod (Gudjónsdóttir et al., 2011). Our results support those findings, with even better correlations obtained for T_{21} parameters, possibly due to a larger dispersion in the T_{22} data as opposed to T_{21}. Thus, it appears to be easier to detect changes in intramyofibrillar water than in intermyofibrillar and extracellular water. There was no significant variation in the total water content until day 30 of the storage period. Because of this, the decrease of the T_{21} population corresponds to an increase in the T_{22} population, and possibly also of other water populations with even larger relaxation times closer to those of free water. Considering the entire storage time, the heterogeneity in the T_{22} population was small, which may have caused strong correlations between WHC and T_{22} (r = 0.99).

The correlation coefficients shown in Table 3 suggest that the instrumental methods of determining moisture, WHC, a_w and pH could be replaced by LF ^{1}H NMR. Of all parameters of LF ^{1}H NMR, the values of $T_{21}$and T_{22} showed the strongest correlation with physicochemical parameters, with a high potential to directly measure moisture, pH and a_w and WHC.

4. Conclusions

Changes in water populations during the storage of Atlantic cod (*Gadus morhua*) could be measured by LF ^{1}H NMR. The relaxation time proved to be a sensitive tool to observe water mobility changes in dry salted cod muscle and offers a useful alternative for investigating water properties in fish. LF ^{1}H NMR relaxation times and physicochemical parameters were strongly correlated suggesting that LF ^{1}H NMR can be used to evaluate fish quality deterioration.

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Table 1. Range of the physicochemical parameters (A_w, moisture, WHC and pH) of Atlantic cod (*Gadus morhua*) according to the days of storage.

Days	A_w	Moisture	WHC	pH
0	$0,745^a$	$49,6^a$	$60,7^a$	$6,57^a$
30	$0,762^a$	$50,1^a$	$59,5^b$	$6,64^b$
74	$0,841^b$	$56,3^b$	49.2^c	$6,86^c$
114	$0,899^c$	$60,1^c$	$43,1^d$	$6,92^d$

a, b, c . . . Different letters in a column indicate significant differences (p<0.01) between treatments (ANOVA).
A_w (water activity)
Water Holding Capacity (WHC)

Table 2. Range of the LF ^{1}H NMR parameters obtained of Atlantic cod (*Gadus morhua*) according to the days of storage.

Days	T_{21}	T_{22}	T_2	A_1	A_2
0	$14,78^a$	$45,82^a$	$18,95^a$	$80,4^a$	$19,6^a$
30	$13,68^b$	$56,22^b$	$17,7^b$	$83,9^b$	$16,1^b$
74	$7,76^c$	$64,41^c$	$12,4^c$	$82,5^c$	$17,5^c$
114	$6,86^d$	$65,55^c$	$11,98^d$	$81,3^d$	$18,6^d$

a, b, c . . . Different letters in a column indicate significant differences ($p<0.01$) within each treatment (ANOVA).

Table 3. Linear correlation matrix for different physicochemical parameters and LF ^{1}H NMR measured during storage.

Variables	A_w	Moisture	pH	WHC	T_{21}	T_{22}	T_2
A_w	**1,000**	0,998	-0,999	0,958	-0,918	0,912	-0,882
Moisture	0,998	**1,000**	-1,000	0,973	-0,939	0,934	-0,908
pH	-0,999	-1,000	**1,000**	-0,970	0,934	-0,929	0,902
WHC	0,958	0,973	-0,970	**1,000**	-0,993	0,991	-0,980
T_{21}	-0,918	-0,939	0,934	-0,993	**1,000**	-1,000	0,997
T_{22}	0,912	0,934	-0,929	0,991	-1,000	**1,000**	-0,998
T_2	-0,882	-0,908	0,902	-0,980	0,997	-0,998	**1,000**

Figure captions

Figure 1

Correlation loadings from PC1 and PC2 from principal component analysis (PCA) of all samples and variables in the study. Two PCs described 99% of the sample variation.

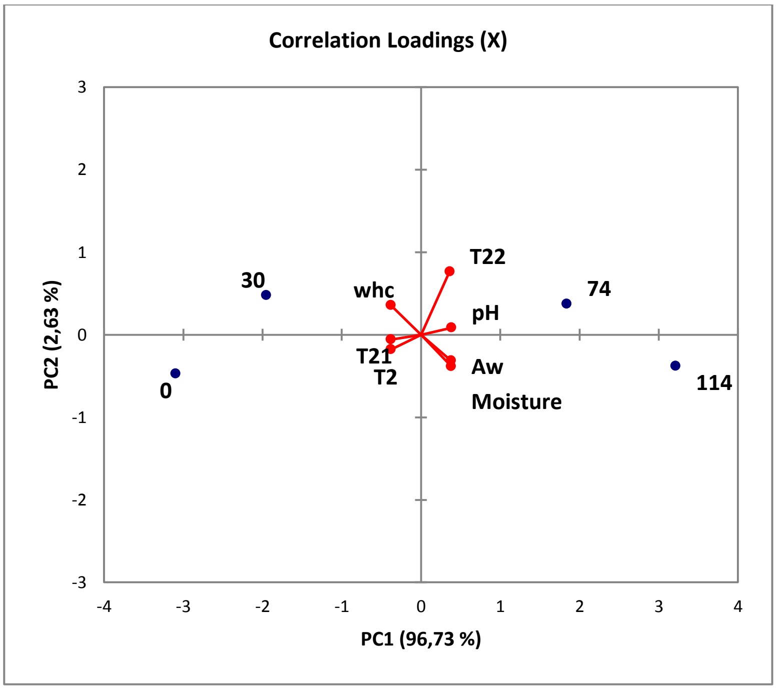

Fig. 1. Correlation loadings from PC1 and PC2 from principal component analysis (PCA) of all samples and variables in the study. Two PCs described 99% of the sample variation.

Figure 2

Partial least squares (PLS) regression of LF 1H NMR versus the physicochemical parameter. Traced lines represent 95% regression confidence limits.

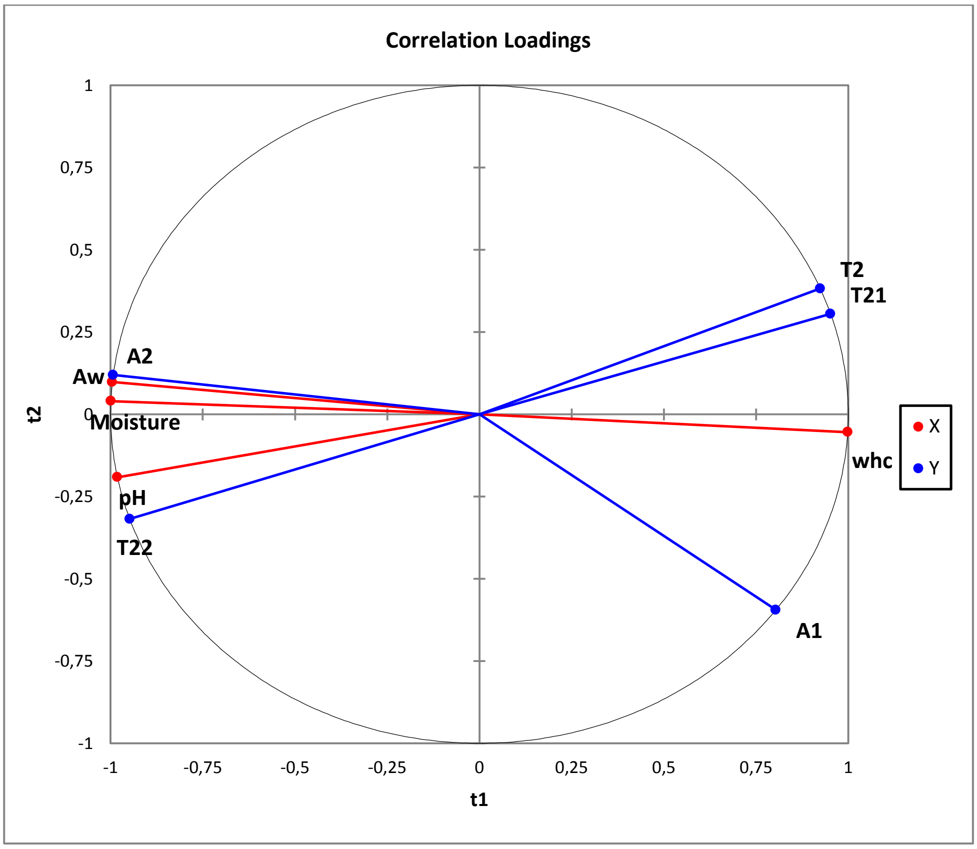

Fig. 2. Partial least squares (PLS) regression of LF 1H NMR versus the physicochemical parameter. Traced lines represent 95% regression confidence limits.

3.4 ARTIGO 4: INFLUENCE OF THE TIME/TEMPERATURE BINOMIAL ON THE HYDROXYMETHYLFURFURAL CONTENT OF FLORAL HONEYS SUBJECTED TO HEAT TREATMENT. Published in Ciência e agrotecnologia, v. 36, n. 2, p. 204 -209, 2012 (Paper IV)

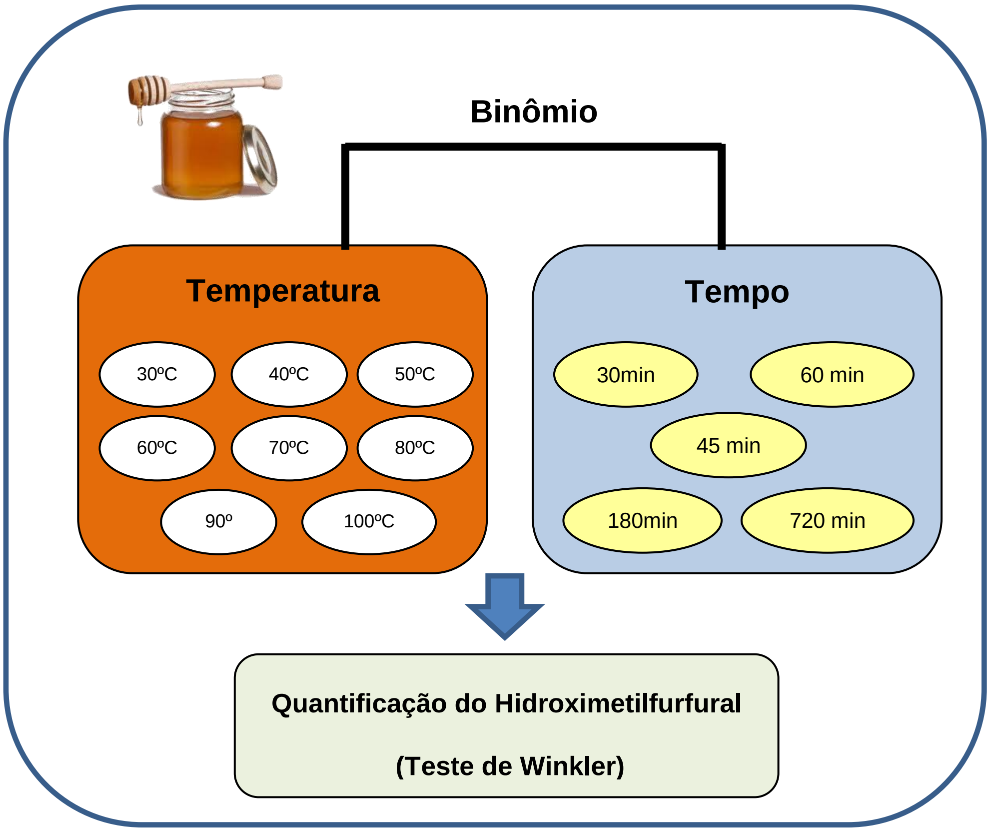

Figura 4. Desenho experimental delineado para desenvolvimento do artigo 4.

Influence of the time/temperature binomial on the hydroxymethylfurfural content of floral honeys subjected to heat treatment

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ABSTRACT

The objective of the present study was to evaluate the evolution of HMF levels in fresh extracted honeys submitted to different temperatures ranging from 30 to 100ºC, during pre-established time intervals (30, 45, 60, 180 and 720 minutes).The test was conducted in floral honey with an initial HMF content of 2.2 mg/Kg. The maximum value recommended by the Brazilian law (60 mg/Kg) was not exceeded in the samples subjected to heating for 30, 45 and 60 minutes regardless of the temperatures used. When the samples were heat treated during 180 minutes at 90ºC the official value was surpassed. The highest values were observed in samples subjected to heating over 720 minutes, and the limit was exceeded at 70ºC. The results obtained indicate that the HMF content gradually increases when the honey is heated at high temperatures for long periods. Therefore, we suggest a process optimization, considering the initial HMF content of the product and standardizing times and temperatures to ensure a good quality of the final product.

Keywords: honey, HMF, time/temperature binomial

INTRODUCTION

Honey is a complex mixture of water, sugars (glucose, fructose, sucrose, maltose), gluconic acid, lactone, nitrogenous compounds, minerals and some vitamins (RAMIREZ et al., 2000). 5-hydroxyimethylfurfural (HMF) is a furanic compound produced by sugar degradation, naturally formed as an intermediate in the Maillard Reaction (AMES, 1992) or from dehydration of hexoses in acid medium (BELITZ and GROSCH, 1999), mainly products with pH value up to 5.0 (DAMODARAN et al., 2010). The presence of simple sugars and water in acid medium favors the formation of this furanic compound (NOZAL et al., 2001).

HMF formation process can be accelerated during the heat treatments applied to honey (KROH, 1994). However, in acid media, HMF can be formed even at low temperatures (LEE & NAGY, 1990), which explains the gradual increase of HMF levels during storage. Some factors, such as the use of metallic containers (WHITE, 1979), honey physico-chemical properties (pH, total acidity, mineral content), the floral origin (ANAM e DART, 1995) and the thermal stress to which the product is subjected in the hive (SPANO et al., 2006),directly influence HMF formation during storage. Due to the factors above mentioned, HMF is considered one of the degradation products more frequently used as honey quality indicator (FALLICO et al., 2004; TOSI et al., 2002), once this product is almost absent in fresh extracted honeys and its concentration increases with time (SPANO et al., 2009), inadequate exposure in retail outlets, aging and improper use of heat to make honey more liquid.

In recent decades, HMF has drawn the attention of the scientific community for its carcinogenic potential for humans. Some studies have shown that this metabolite can be converted *in vivo* to 5-sulfooxymethylfurfural (SMF), a genotoxic compound (SURH et al, 1994). In addition, at high concentrations, HMF is cytotoxic, causing irritation to eyes, upper respiratory tract, skin and mucous membranes (ULBRICHT et al., 1984 & BRUCE et al., 1993). For this reason, the *Codex Alimentarius* and the European Commission have set a maximum HMF level for honey of 40 mg/Kg, except for honeys coming from tropical countries and honeys with low enzyme levels, the HMF limit of which was set in 80 and 15 mg/Kg respectively (CODEX, 1981 & EUROPEAN COMMISSION, 2002). The Brazilian legislation recommends a maximum limit of 60 mg/Kg for all kinds of honey (BRAZIL, 2000).

The amount of HMF detectable in honey is directly related to the intensity of the heat and the exposure time applied during processing. The objective of the present study was to evaluate the evolution of HMF content in fresh extracted honeys, subjected to different temperatures ranging from 30 to 100ºC, over pre-established time intervals (30, 45, 60, 180 and 720 minutes).

MATERIALS AND METHODS

Honey samples

Honey samples were directly obtained from an apiary located in the mountain region of Rio de Janeiro, in the municipality of Teresopolis, a region characterized by diverse vegetation with altitude tropical climate and average annual temperature of 16±2ºC. Approximately 6 Kg of multifloral honey were fractionated in 120 glass bottles with 20 mL capacity and stored at 20±1ºC.

Treatments

In order to evaluate the effects of time and temperature on the variation of HMF levels in the samples of floral honey, heat treatments were performed, in triplicate, by immersion in a thermostatic bath with periodic shaking, set at the following temperatures: 30, 40, 50, 60, 70, 80, 90 and 100ºC. During this stage, the samples were kept at those temperatures during 30, 45, 60, 180 and 720 minutes. Temperature was monitored with a thermocouple introduced at the geometrical center of the bottles. The time was measured from the moment the temperature reached the value established in the methodology. Then, the samples were cooled by immersion in cold water at 4±2ºC and immediately analyzed.

Physico-chemical analysis

The samples of honey were submitted to physico-chemical analysis in order to verify their quality before undergoing heat treatment. The following techniques were used: determination of pH, fixed mineral residue, insoluble matter, reducing and non-reducing sugars, Lund reaction, acidity analysis, Fiehe reaction.

HMF content determination

The content of HMF in the samples was determined by Winkler spectrophotometry method. Five grams of honey were weighted, dissolved in distilled water and transferred to a 25mL volumetric flask and make up to the mark. Immediately after preparation a 2 mL aliquot was transferred to a test tube and 5mL of 10% p-toluidin solution in isopropanol and 1mL of 5% barbituric acid aqueous solution were added. In another test tube (reference) was added 2mL of honey solution, 5mL of p-toluidin solution and 1mL distilled water. The reagents were used in up to 2 minutes after preparation in order to ensure the stability of the solutions. The absorbance at 550 nm was determined using a spectrophotometer. The honey used contained an initial HMF value of 2.2 mg/Kg before heating.

Statistical Analysis

Data were statistically analyzed using the two-way analysis of variance (ANOVA), aiming at identifying differences in HMF content in honey caused by the interaction of time/temperature variables. Multiple regression was applied with HMF content as dependent variable and the sample immersion time in the hydrostatic bath and the temperature of the bath as independent variables. All the analyses were performed using a statistic package available in the market (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, California, USA).

RESULTS AND DISCUSSION

The values obtained during the experiment are presented in tables and figures that summarize the influence of the heat treatments on HMF content in the samples of floral honey analyzed. HMF content significantly varied ($P<0.001$) with the increase of temperature and immersion time to which the samples were subjected. The regression coefficient ($r=0.98$), indicated that only 2% of the regression variances do not depend on the variables studied. It was observed that HMF content increases slowly up to 70ºC and, above this temperature, its production rate increases in a more significant way, mainly at temperatures above 80ºC. The HMF content increase is more evident when the immersion time in the hydrostatic bath increases, reaching its highest value in the samples exposed to 100ºC for 720 minutes, which presented an average value of 101.24 mg/Kg (Figure 1).

The HMF content variation was small in the treatments at 30, 40 and 50ºC for up to 180 minutes, varying from 4.46 mg/Kg at 30ºC during 30 minutes to 15.28 mg/Kg at 50ºC for 180 minutes, indicating that the use of those temperatures in honeys with low initial HMF content does not cause a significant increase of this compound. In general, it can be observed that honeys heat treated at 40 and 50ºC for up to 180 minutes does not present a significant difference of HMF formation ($P<0.05$). Karabournioti and Zervalaki (2001) reported a slightly significant increase of HMF content in orange honeys heat treated at 35ºC, 45ºC, 55ºC and 65ºC for 24 hours, from 2.25 mg/Kg (without heating) to 3.45, 3.75, 4.35 and 19.00 mg/Kg, respectively and concluded that mild temperatures up to 55ºC, do not influence the increase of HMF regardless of the exposure time.

The effects of heating on HMF increase can be observed when the exposure time is longer, the time variable being the most significant factor in increasing HMF content, when temperatures between 30 and 90ºC are considered. The results show that there was no significant difference ($p>0.05$) on HMF levels in samples submitted to temperatures of 30, 40, 50, 60, 70 and 80ºC for 30 minutes. The values increased from 2.2 mg/Kg (fresh extracted honey) to 4.46, 5.68, 6.96, 7.12, 8.88 and 10.68 mg/Kg, respectively. Samples heated at 80, 90 and 100ºC for 30 minutes, significantly differed ($P<0.05$) from samples kept in thermostatic bath for 45 minutes confirming the trend of increased levels of HMF when the heating time increases.

The appearance of honey plays an important role on its commercial acceptance, once consumers demand a fluid, non-crystallized product. Fresh extracted honey is liquid. However, it may crystallize during storage at higher or lower speed depending on several factors such as origin (botanical and geographical), temperature, moisture content and sugar content (BATH & SINGH, 1999; PIRO et al. 1996). In order to delay the natural crystallization process and ensure stability during its shelf-life, fresh honey is usually submitted to heating before being packed, with the purpose of dissolving sugars and destroying yeasts (BATH & SINGH, 1999; TOSI et al., 2002). The results of the present study indicate that honey should be heated at mild temperatures, between 40ºC and 50ºC, heat treatments above 90ºC are not recommended.

According to Jeanne (1985) de-crystallization of finely crystallized honeys requires different times and temperatures, for example, it is recommended that a 20 Kg recipient is heated at 40º C during 24 hours, and the author suggests a time/temperature binomial of 72 h at 50ºC for a 300 Kg recipient. However, the present study showed that high contents of HMF (2.15 and 32.63 mg/Kg, for samples exposed to 40 and 50ºC, for 12 hours, respectively), can be obtained when heat treatments are applied during long periods. Although the maximum level of HMF was not exceeded at temperatures up to 50ºC, the value was close to that recommended by international legislation, which could limit the international trade of samples submitted to heating. An effective temperature control should be kept during honey de-crystallization stages, because a considerable HMF increase may occur when heating at 70ºC as shown in the present study. After 720 minutes heating, the HMF content was 64.37 mg/Kg, a value that exceeds the value allowed by the Brazilian law (60mg/Kg) (BRAZIL, 2000). Fallico et al. (2004), observed a significant increase of HMF content in eucalyptus honey subjected to 70ºC, for 96 hours, from an initial value below detection level to 513 mg/Kg, in agreement with data obtained in the present study.

At high temperatures, such as 80 and 90ºC, a gradual increase was observed that significantly varied ($P<0.01$) with heating time, which was the factor that mostly influenced the increase of HMF content at these temperatures of samples that when heated at 90ºC presented HMF levels of 29.55 and 66.96 mg/Kg, after 60 and 180 minutes heat treatment, respectively. However, Turhan et al. (2008) presented different results. They reported low HMF contents in floral honeys subjected to 75 and 90ºC heat treatments for up to 90 minutes. Those authors concluded that there was no significant increase of HMF in floral honeys heated at 90ºC for up to 90 minutes, obtaining a maximum HMF content of only 11.24 mg/Kg.

It should be enhanced that, at 100ºC, the formation of HMF presented a different behavior than at other temperatures, and, in this case, temperature was the factor that most significantly ($P<0.01$) influenced the increase of HMF, since there was an increase at all the times studied. In the same way, Turhan et al. (2008), described an increase of HMF content in floral honeys heated at 100ºC for 30, 45, 60, 75 and 90 minutes, from 0.62 mg/Kg in samples without heating to 13.99, 26.4, 37.98, 55.41 and 73.78 mg/Kg, respectively. These authors observed a low HMF content in honeys subjected to heat treatments up to 90ºC, demonstrating that extreme heating significantly accelerates HMF formation.

Tosi et al. (2002) studied the effects of applying temperatures between 100 and 160ºC in short time intervals, varying from 14 to 60 s. The authors concluded that temperatures above 140ºC, even when applied during short time intervals, considerably increased HMF contents, which reached values above those recommended by international standards, demonstrating the influence of temperature on the increase of HMF content even in short time intervals. In the present study, we observed an increase of HMF content in samples submitted to heat treatment at 100ºC, regardless the heating time, significantly differing ($P<0.05$) at all levels, reinforcing the relevance of the study and in accordance with the above mentioned authors. After 60 minutes heating, HMF concentration was 48.19 mg/Kg, exceeding the limits recommended by the European legislation. Such increasing trend was maintained up to 720 minutes, when HMF concentration reached the highest levels, above100 mg/Kg.

The maximum value recommended by the Brazilian legislation of 60 mg/Kg (BRAZIL, 2000), was not exceeded in the samples subjected to heating for 30, 45 and 60 minutes, regardless the temperature used. However, increasing the time that the samples were kept in the thermostatic bath to 180 minutes, the samples exceeded the legal values at temperatures above 80ºC. It was also observed that samples heated for 180 minutes significantly differed ($P<0.05$) of samples heated for 720 minutes regardless the temperature used.

The samples heated during a 720- minute period did not exceed the acceptable limit of 60 mg/Kg, when the temperature was 70ºC and, the samples submitted to 40ºC, presented an average HMF content of 27.15 mg/Kg. Some authors observed that the increase of HMF is not related only to heating, and can occur during long storage periods, regardless the temperature. Khalil et al. (2010) studied HMF concentration in Malasian honeys stored during more than a year. They concluded that honey samples when stored during 12 to 24 months presented HMF concentrations that exceeded the recommended levels, reaching values of 118.47 and 1139.95 mg/Kg, respectively. Kalábová et al. (2003) demonstrated that the content of HMF gradually increases during storage, eventually reaching values above those recommended by current legislation. Therefore, the time/temperature binomial used for honey processing should be as low as possible, considering that HMF increase may be influenced by factors other than exposure to adverse temperatures.

However, it is important to emphasize, that, in this study, honey was collected at Teresópolis municipality, a region characterized by altitude tropical climate, with cold, dry winters and template, humid summers, with annual average temperature of 16±2ºC and relative humidity of the air of 84%. Although the European legislation is more flexible with products from tropical regions, allowing contents of up to 80 mg/Kg, the floral honey acquired for this study was not exposed to high temperatures in the hive, which explains the initial low content of HMF (2.2mg/Kg). Thus, we used 40 mg/Kg and 60 mg/Kg, as references, the first is the value recommended by the Codex Alimentarius (CODEX, 1981) and the European Union Council (EUROPEAN COMMISSION, 2001) for honeys of non-tropical regions and the second the value recommended by the Brazilian legislation (BRAZIL, 2000). These values are represented in Figure 1, by grey and black horizontal lines.

CONCLUSIONS

We concluded that HMF content significantly increases with the exposure to high temperatures during a long time. Considering the positive correlation between the thermal treatment and the increase of HMF content in fresh extracted honeys, we suggest that HMF content is used as an indicator of honey quality loss caused by heating. Honeys with low initial HMF content may be submitted to temperatures up to 90ºC for periods up to 60 minutes, for de-crystallization. However, for longer periods of time, up to 180 minutes, the temperature should not exceed 70ºC. Since honey overheating is not necessary for the filling process, mild temperatures should be used during limited times to reduce viscosity and prevent crystallization, without causing a significant increase of HMF content.

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Table 1. Variation of the concentration of Hydroxyimethylfurfural (mg/Kg) in honey samples as a function of temperature and time of immersion in a thermostatic bath

Temperature	Immersion time				
	30 min	**45 min**	**60 min**	**180 min**	**720 min**
30ºC	4.46±0,15^{Aa}	4.77±0,12^{Aa}	5.04±0,90^{Aa}	9.97±0,43 ^{Ba}	16.18±0,37^{Ca}
40ºC	5.68±0,83^{Aa}	5.82±0,32^{Aa}	6.58±0,53^{Aa}	14.05±0,68^{Bb}	27.15±0,18^{Cb}
50ºC	6.96±0,13^{Aa}	7.39±0,22^{Aa}	8.18±0,14^{Aa}	15.28±0,80^{Bb}	32.63±0,37^{Cc}
60ºC	7.12±0,22^{Aa}	7.33±0,16^{Aa}	10.97±0,10^{Aa}	18.32±0,29^{Bb}	47.19±3,19^{Cd}
70ºC	8.88±0,37^{Aa}	9.61±0,43^{Aa}	15.24±0,19^{Bb}	20.96±0,11^{Cb}	64.37±0,11^{De}
80ºC	10.68±0,28^{Ab}	15.62±0,24^{Bb}	21.35±0,30^{Cc}	41.03±0,08^{Dc}	85.10±0,26^{Ef}
90ºC	13.54±1,10^{Ac}	18.36±0,24^{Bc}	29.55±0,30^{Cd}	66.96±0,28^{Dd}	94.23±0,56^{Eg}
100ºC	19.31±2,40^{Ad}	27.48±0,32^{Bd}	48.19±1,35^{Ce}	82.79±3,23^{De}	101.24±0,35^{Eh}

Figure captions

Figure 1

Fig. 1: Graph of the results of the determinations of HMF content according to temperature and time of immersion of honey samples.

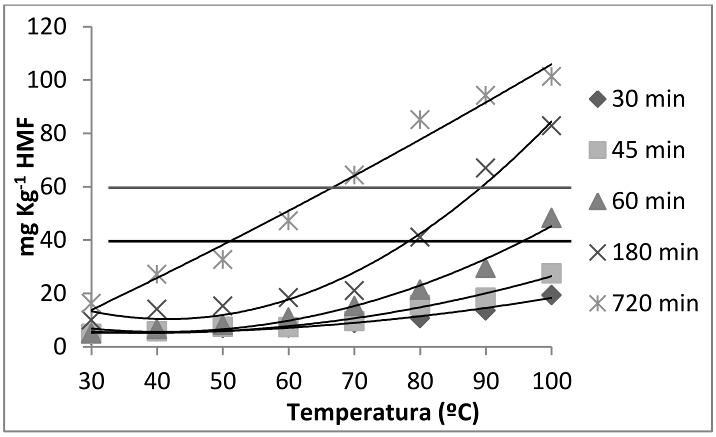

Figure 2: Graph of the results of the determinations of HMF content according to temperature and time of immersion of honey samples.

3.5 ARTIGO 5: Detection of honey adulteration by high fructose corn syrup and Saccharose syrup by using important biochemical properties of honey. Under review in Italian journal of food science (Paper V)

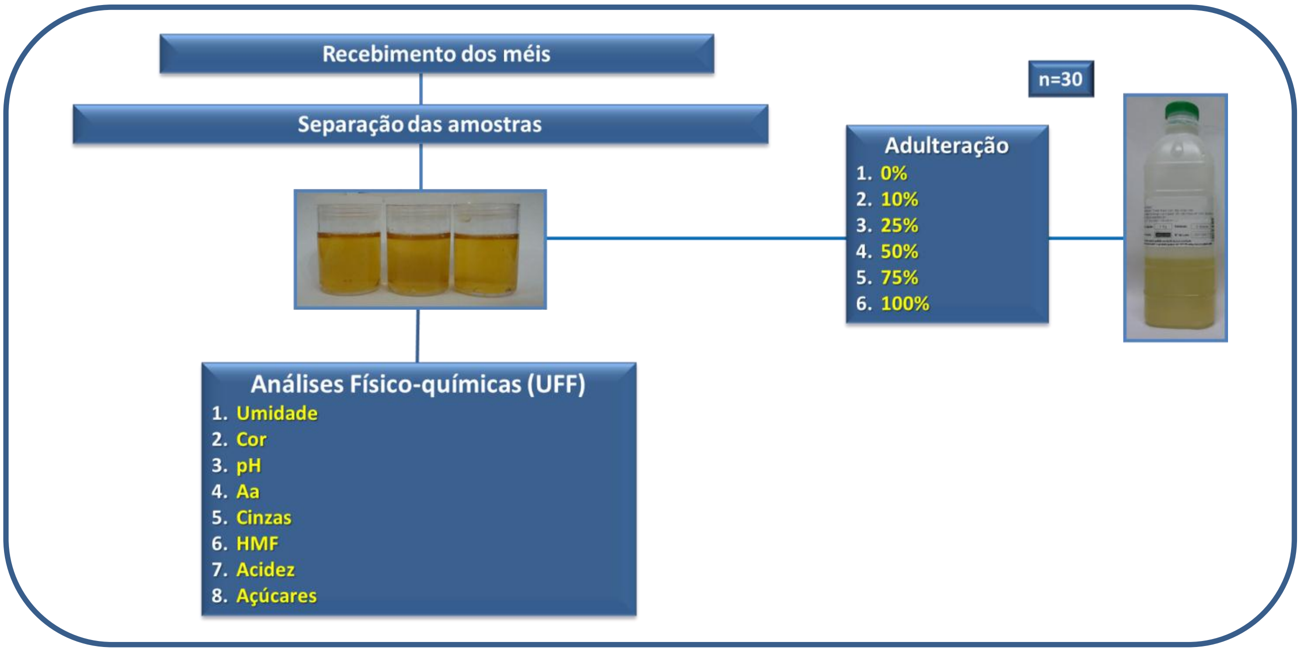

Figura 5. Desenho experimental delineado para desenvolvimento do artigo 5.

Detection of Brazilian honey adulteration by high fructose corn syrup and Saccharose syrup using biochemical properties of honey

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ABSTRACT

Honey adulteration is a complex problem, which currently has a significant economic impact and undeniable nutritional and organoleptic consequences. In this study, pure honey was intentionally adulterated with high-fructose corn syrup (HFCS) and saccharose syrup (SS) at levels of 10%, 25%, 50%, 75%, 90% and 100% (w/w). The physicochemical properties were determined for pure and adulterated honey: moisture, water activity (a_w), pH, free acidity, HMF, sugar contents and colour. The results for moisture, a_w, protein, HMF and coulor can be taken as key differential criteria to detect the adulteration of honey with HFCS and SS in contrast to pure honey. In distinction, ash, pH, free acidity and sugar contents were poorly significantly positively correlated to distinguee for pure and adulterated honey. Accordingly, such quick tests can be applied as good indicators for detecting the adulteration of honey with HFCS and SS at levels ranging from 25% to 90%.

KEYWORDS: Honey; high fructose corn syrup; saccharose syrup; physicochemical properties

1. Introduction

The Brazilian annual nectar honey production was estimated at about 40,000 tons for the year 2010 (latest available data). The increase of honey production, which doubled from 18,000 tons in 2000 to 40,000 tons in 2010, ranks Brazil at the 11th position of world-wide producers and has made the country the fifth largest exporter (IBGE, 2010). As a result of the high nutritional value and the unique flavour characteristics of natural bee honey, its cost is much greater than that of any other sweetener and it can, therefore, be a target of adulteration for economic gains (Sivakesava & Irudayaraj, 2002).

Bee honey is a unique sweetening agent that can be used by humans without processing, and it provides significant nutritious and medical benefits (Meda et al., 2005; Schramm et al., 2003; Perez et al., 2007). Because of its nutritional and medicinal value, honey continues to be a popular food. Unfortunately, honey can easily be adulterated with various cheaper sweeteners, such as refined cane sugar beet sugar, high fructose corn syrup, saccharose syrup and maltose syrup, resulting in higher commercial profits (Ruiz-Matute et al., 2010). HFCS offers a variety of advantages to commercial users. Because HFCS is a liquid, as opposed to being crystalline, such as sucrose, the sweetener offers advantages in storage, transportation, and distribution logistics for industrial users (Long, 1991; Buck, 2001), although some saccharose syrup is delivered to companies as a liquid.

The composition of honeys depends highly on the type of flowers utilized by the bee as well as climatic conditions (Abu-Tarboush, Al-Kahtani, & El-Sarrage, 1993, Gheldof & Engeseth, 2002). The physical properties and chemical composition of honey from different sources have been published by many scientists (Belay et al., 2013; Chua et al., 2012; Escriche et al., 2014; Estevinho et al., 2012; Ouchemoukh et al., 2007; Ribeiro et al., 2012; Silva et al., 2013; Unal & Kuplulu, 2006). Quality parameters are considered useful to detect product adulteration during manufacture (water and sugar or syrup addition) and also to confirm a hygienic production and efficient storage of honey (Andrade et al., 1999; Fernández-Torres et al., 2005). To our knowledge, the scientific data which uses physicochemical properties for detection honey adulteration from Brazilian honeys is very limited. The physicochemical analyses are a practically important, especially in developing countries, where sophisticated analytical instruments are not available for routine analysis.

The aim of this study was to determine the physicochemical properties of honey samples to be able to make use of these properties to distinguish between the adulterated honey (high fructose corn syrup and saccharose syrup) and pure blossom Brazilian honey samples.

2. Materials and methods

2.1. Samples

This study was carried out at a farm in the mountainous region of Rio de Janeiro in the South-East of Brazil. Bee colonies were kept in Teresópolis (22º 24' 44" S, 42º 57' 56" W). Pure blossom honey samples (n=30) were collected by beekeepers and stored in the dark in screw-cap jars at room temperature (18-23ºC) from the time of collection to spectral analysis (a maximum of 2 weeks after extraction from the hives).

2.2. Sample collection and treatment

To determine the effect of adulteration on the physicochemical properties of honey, two experimental mixtures were prepared using pure blossom honey samples and high fructose corn syrup, another one using pure blossom honey samples and saccharose syrup. Pure model mixtures were prepared by adding high fructose syrup and saccharose syrup to honey samples at a ratio of 0, 10, 25, 50, 75, 90 and 100% by weight. After adding the high fructose syrup and saccharose syrup, samples were kept in a water bath at 35ºC for 20 min and stirred for 1 min followed by cooling in an ice bath.

2.3. Physicochemical analysis

2.3.1 Total sugar content and moisture content determination

The total sugar content and moisture content were determined in triplicate by refractometry according to AOAC methods (AOAC, 2005) using an Abbe-type refractometer at 20ºC by adding the correction factor of 0.00023/ºC. Percentages of total sugar content and moisture content in honey samples were obtained from refractive index.

2.3.2. pH

The honey pH was measured with a pH electrode connected to a pH meter (Digimed® Model DM-32, São Paulo, Brazil). The electrode was immersed in a suspension made by mixing for a 10% (w/v) solution of honey of milli Q water (AOAC, 2005).

2.3.3. Hydroxymethylfurfural (HMF)

Hydroxymethylfurfural (HMF) was determined spectrophotometrically as outlined by Harmonization methods of International Honey Commission (IHC) (Bogdanov et al., 2000).

2.3.4. Ash content

The ash contents was determined with a weighed sample was ignited in a muffle furnace at 550ºC to a constant weight for carbonization (AOAC, 2005).

2.3.5. Water activity (a_w)

The water activity was measured with an instrument Pawkit, Decagon Devices from USA. A small aliquot of honey sample weighing approximately 1 g was used for determining water activity (a_w) using a water activity meter. This measure the water activity of the sample based on its equilibrium relative humidity (ERH). The measurements were performed in triplicates for each sample and the mean was determined.

2.3.6. Color

The intensity of the color was measured with a Minolta type CR-400 colorimeter (Konika Minolta®, Tokyo, Japan) according to the manufacturer’s instructions. The instrument records *L*** (lightness—intensity of white color), *a*** (redness—intensity of red color) and *b*** (yellowness—intensity of yellow color) values.

2.5. Statistical analysis

The analyzes were carried out in duplicate and the results are expressed as mean values and standard deviation (SD). The statistical differences represented by capital letters (Table 1 and 2) were obtained through one-way analysis of variance (ANOVA) followed by Tukey's at 99% of confidence level ($p < 0.01$).

3. Results and discussion

The quality parameters of honey samples are listed in Table 1 and 2, for the three groups of honey samples (unadulterated and adulterated with high fructose corn syrup and saccharose syrup). Adulteration in honey resulted in modifications of the honey samples' quality parameters, which were significantly affected ($p < 0.05$).

3.1. Physical analysis

3.1.1 Moisture content

Moisture contents of pure honey samples were below 20%, the maximum value allowed by Brazilian law (Brazil, 2000), which means that the fermentation ability is low. Honey samples adulterated with HFSC and SS had a significant ($p < 0.05$) higher moisture contents, in all percentages of adulteration. Sugars are dominating contents of the honeys dry mass ranging from 80% (Maurizio, 1966) to 99% (White, 1976) and the high sucrose content of HFSC and SS may be an explanation of its high moisture content. Moisture content depends on the botanical origin of the sample, the degree of ripeness, processing techniques and storage conditions (Finola et al., 2007). Higher moisture content could lead to undesirable honey fermentation during storage caused by the action of osmotolerant yeasts resulting in the formation of ethyl alcohol and carbon dioxide. The alcohol can be further oxidized to acetic acid and water resulting in a sour taste (Chirife et al., 2006). The moisture content of pure honey (17.60%) increased gradually as percentage of HFCS and adulterants were raised. However, the 50% adulteration with HFCS level possessed a moisture content of 20.20%, which is more than the critical moisture level (20%) from Brazilian law. In the samples with SS these level was exceeded in 25% of adulteration (20.60%).

3.1.2. Water activity

The water activity (a_w) of the honey samples varied from 0.51 for pure honey to 0.76 for adulterated honey with SS. Some variations were traced with regard of pure honey and adulterated honeys with HFCS and SS. The point of interest is that the water activity increased gradually as the HFCS and SS percentage was raised (Table 1 and 2). Our results are quite similar to those of unadulterated Greek honeys for which the a_w values ranged from 0.53 to 0.67 (Lazaridou et al., 2004). The water activity is an important factor, which governs the food stability by preventing or limiting microbial growth. The osmotolerant yeasts are able to grow at a minimal water activity of 0.6 (Chirife et al., 2006). The adulterated honey samples, which had the highest moisture content was found to have the highest water activity. Our results are different for the Indian honeys, which had inverse correlation was found between the water activity and moisture contents (Saxena et al., 2010). Such observations are occasionally observed in certain honey samples probably due to the variation in the constituents and/or levels of individual sugar components (Abramovic et al., 2008).

3.1.3. pH

In general, honey is acidic in nature, irrespective of its geographical origin (Ribeiro et al., 2013a). The pH of honey can be influenced by the different sources of nectar, soil, or combination of plants for honey composition, by the action of glucose oxidase, the action of bacteria as the honey ripens, and also the amount of minerals present in the honey (Finola et al., 2007, Ribeiro et al, 2013). Honey pH values are of great importance during storage, as they influence the texture, stability and shelf-life of honeys (Baroni et al., 2009; Downey et al., 2005; Terrab et al., 2004). Most bacteria grow in a neutral and mildly alkaline environment, while yeasts and molds are capable of growth in an acidic environment (pH = 4.0–4.5) (Conti, 2000). The pure honey under study had quite similar pH values ranging from 3.08 to 3.15 (Table 1). These values fall within the range usually observed for natural honeys (Downey et al., 2005; Khan et al., 2006), which may even reach pH 5.0, which can be considered to be of low purity and low quality. Upon the adulterated honey showed remarkable changes in all of its physical properties. The HFSC and SS honey samples considered in this study exhibited a relatively high pH ranging from 3.30 to 4.80 for HFSC and 3.80 and 5.90 for SS (Table 1 and 2). The pH increase was significant ($p < 0.05$) in the samples from HFCS 25% adulterated. For the samples SS this result was even more significant because it was possible to observe difference in pH from 10% to fraud.

3.1.4. Free acidity

Free acidity it was inversely proportional to the percentage of HFCS and SS used as an adulterant. The results was highest (23.8 meq/kg) in natural honey, than 100% HFCS (19.2 meq/kg) and 100% SS (16.4 meq/kg). The lowest acidity shown by the adulterated honeys with HFSC and SS can be connected with more dissociated organic acids contained it (Popek, 2002). Also free acidity (Table 1 and 2) was within the wide range of values reported for natural honeys (Khan et al., 2006; Nanda et al., 2003; Terrab et al., 2004) being poorly significantly positively correlated to distinguee for pure and adulterated honey. The free acidity of the samples studied not exceed the limit value (50 meq/kg) permitted by Brazilian legislation for natural honeys (Brazil, 2000).

3.1.5. Sugar

The total sugar and the total reducing sugar content in the honey samples adulterated with HFCS and SS ranged from 49.5% to 71.7% and 47.2% to 70.0%, respectively (Table 1). The results indicated that the majority of sugars in honey samples are reducing sugars. The mean of sucrose content in the pure honey was 1.70%. The adulterated honeys samples had sucrose levels above 5% which is the maximum prescribed limit as per the Codex standard and Brazilian law (Codex Alimentarius, 2001; Brazil, 2000). Higher sucrose content could be attributed to reasons such as overfeeding of honeybees with sucrose syrup, adulteration, or an early harvest of honey, wherein sucrose has not been fully transformed into glucose and fructose (Anklam, 1998; Azeredo et al., 2003; Guler et al., 2007). The level of sucrose differs according to the maturity degree and origin of the nectar compound of the honey. Cantarelli et al. (2008) reported sucrose content in pure honey samples at the average of 4.05%. In another study with pure honey, sucrose was detected between 2.21% and 5.52% (Rodriguez et al., 2004). Our findings showed the adulterated samples of HFCS and SS shown highest level of sucrose content in all samples ranged from 2.95% to 9.55% and 3.95% to 12.25%, respectively.

Reducing sugars, which include mainly glucose and fructose, are the major constituents of honey (Küçük et al., 2007). A pure honey from this study had 72.25% of combined glucose and fructose content. The samples adulterated increase on results of reducing sugars; however, the result was not significantly different ($p < 0.05$) for samples of pure honey in the samples adulterated.

3.1.6. Hydroxymethylfurrural

Overheating of honey samples during processing or storage for very long periods could lead to the conversion of sugars to hydroxymethylfurfural. A low level of hydroxymethylfurfural (HMF) is an indicator of the freshness of honey (Ribeiro et al, 2012). In a parallel study, the HMF content of honey samples has been determined. In pure honey samples the HMF content was 12.00 mg/kg which considerably lower than internationally recommended limit of 80 mg/kg (Saxena et al., 2009). HMF formation results from the acid-catalysed dehydration of hexose sugars with fructose being particularly susceptible to this reaction. Stability of sugar molecules explains the faster rate of HMF formation from fructose than from glucose because fructose enolizes rapidly (Isbell et al., 1969; Kuster, 1990; Lee & Nagy, 1990). The chemical properties of honey, such as pH, total acidity and mineral content, influence the formation of HMF (Anam & Dart, 1995; Bath & Singh, 1999; Hase, Suzuki, Odate, & Suzuki, 1973; Singh & Bath, 1997). The increases on HMF content of samples adulterated with SS may be attributed to the heating process for processing (Ribeiro et al, 2012). On the other hand, samples adulterated with HFSC gave the most increased formation of HMF (76.80 mg/kg) of all the samples because it contained more fructose reactant and had the lower pH (3.30 to 4.70). In the presence of nitrogen-containing molecules, the competing mechanism for the formation of HMF would be through the formation of Schiff base-type intermediates, such as with Amadori rearrangements, as is found in nitrogen-containing products (Bradford, 1976). According to Kuster (1990), fructose is about 40 times more reactive than glucose in forming HMF. The HMF content increased gradually as the HFCS percentage was raised. This is in accordance with the observation of Abdel-Aal (1993), who reported that the level of HMF in fresh honey is normally very low but increased with the addition of HFCS.

3.1.7. Ash content

The percentage ash content is an indicator of the mineral content. It is considered as a quality criterion indicating the possible botanical origin of honey. The wide variability of honey composition is also reflected in the ash content (Baroni et al., 2009). The higher ash content of HFCS (0.17 to 0.31 g/100g) and of SS (0.19 to 0.39 g/100g) may be related to the residues of acidic and alkaline solutions added to it. This increment was such that the ash for honey adulterated with 75% SS was more than twice that for pure honey (Table 2). However, ash content in each of the pure honeys samples and adulterated samples was appropriate for the limit allowed in flower honeys, 0.6%, in Brazilian law (Brazil, 2000). The variability in the ash content of honeys could be due to harvesting processes, beekeeping techniques and the material collected by the bees during the foraging on the flora (Finola et al., 2007), but in our studies the increases on ash content was observe in all samples adulterated with SS and HFCS.

3.1.8. Colour measurement

The color characteristics are presented in Table 1 and 2 with the values for *L** (lightness—intensity of white color), *a** (redness—intensity of red color), and *b** (yellowness—intensity of yellow color). Honey samples having an *L** value > 50 are considered lighter, and samples having an *L** value ≤ 50 are dark. Based on this classification, the pure honey samples studied here were relatively dark, with *L** mean values 48.17. In the HFCS and in SS samples, the values of parameter *a** were positive, indicating a large proportion of red colours. All adulterated samples showed positive values of parameter *b**, characteristic of yellow colours. The value was highest in adulterated honey with HFCS, and lowest in aloe honey with SS.

Ozcan et al. (2006) reported that a honey adulterated with SS showed the highest mean for colour.

3.1.9. Protein

The pure honey samples studied were within the legal standards permitted by Brazilian legislation for natural honeys (Brazil, 2000). The adulterated honey with 100% HFCS and SS exhibited no levels of protein, which is also characteristic just of natural pure honeys. Among the nitrogen substances contained in honeys, enzymes and free essential amino acids, mainly from the facial glands of bees, play an important part. In our studies, was observed the decreases on levels of protein with the increased gradually as percentage of both adulterations.

4. Conclusions

This work has shown that it is possible to discriminate between pure honey and adulterated honeys with high fructose corn syrup (HFSC) and saccharose syrup (SS), with acceptable classification success rates. This discrimination is possible both for HFSC samples and for SS samples, although for the SS samples it becomes more difficult to distinguish between the pure honey and adulterants. Adulterated honey produces changes in the samples, which lead to marked changes in the physical and chemical composition. It can be concluded that moisture, a_w, HMF and pH can be taken as key differential criteria to detect the adulteration of honey with HFCS and SS in contrast to pure honey.

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4 CONSIDERAÇÕES FINAIS E SUGESTÕES

Com base nos trabalhos desenvolvidos, pode-se concluir que a LF ^{1}H NMR é um método com alto potencial para aplicada como ferramenta analítica na pesquisa de qualidade e processamento de produtos de origem animal. A citada técnica ainda é pouco pesquisada no âmbito dos alimentos, e suas aplicações ainda não são plenamente exploradas. As medições dos tempos de relaxação T_2 e seus binômios mostraram-se eficiente, contribuindo para uma melhor compreensão da dinâmica da água diferentes produtos de origem animal, tais como no mel e musculatura de pescados. Os componentes de relaxação T_2 mostraram significativas correlações com os métodos convencionais de análises físico-químicas da água, tais como a umidade, capacidade de retenção de água e pH.

A LF ^{1}H NMR gerou informações úteis sobre a classificação e a qualidade das matérias-primas estudadas, demostrando ser uma tecnologia importante devido as suas características não invasivas, de alta reprodutibilidade e sensibilidade, sendo um método rápido, para investigar a mobilidade da água em uma grande variedade de produtos de origem animal. Este estudo fornece dados preliminares que podem ser ampliados para diferentes matrizes, sugerindo dados relevantes para a tecnologia de alimentos. Na ciência de alimentos, ainda existe algumas barreiras para utilização de instrumentos sofisticados, principalmente, devido ao alto custo, e as questões de segurança relacionadas com a manutenção dos equipamentos. A LF ^{1}H NMR é relativamente mais acessível a pesquisadores de alimentos devido ao menor custo do equipamento e manutenção mais simplificada, quando comparado a outros equipamentos de RMN.

Neste contexto, ainda é possível concluir que os métodos convencionais de análises físico-químicas avaliados neste estudo, tais como o teste de Winkler para quantificar o HMF em méis, são métodos eficazes, entretanto possuem limitações devido ao tempo gasto para preparação e medição das amostras, a necessidade de grande quantidade de amostra e a utilização de reagentes com alto grau de toxicidade.

Ademais, baseando-se nos resultados obtidos, sugere-se a realização de estudos futuros em LF ^{1}H NMR, visando construir para determinação rápida de vários parâmetros de qualidade, que pode então ser facilmente aplicados como ferramenta de controle de qualidade em diversos produtos. A otimização da técnica de LF ^{1}H NMR é necessária visando simplificar o processo e torná-lo acessível tanto para os gestores de qualidade na indústria, que supervisionam as linhas de processamento e classificação de produtos de origem animal, assim como para comunidade científica no desenvolvimento de técnicas rápidas e seguras de avaliação dos produtos.

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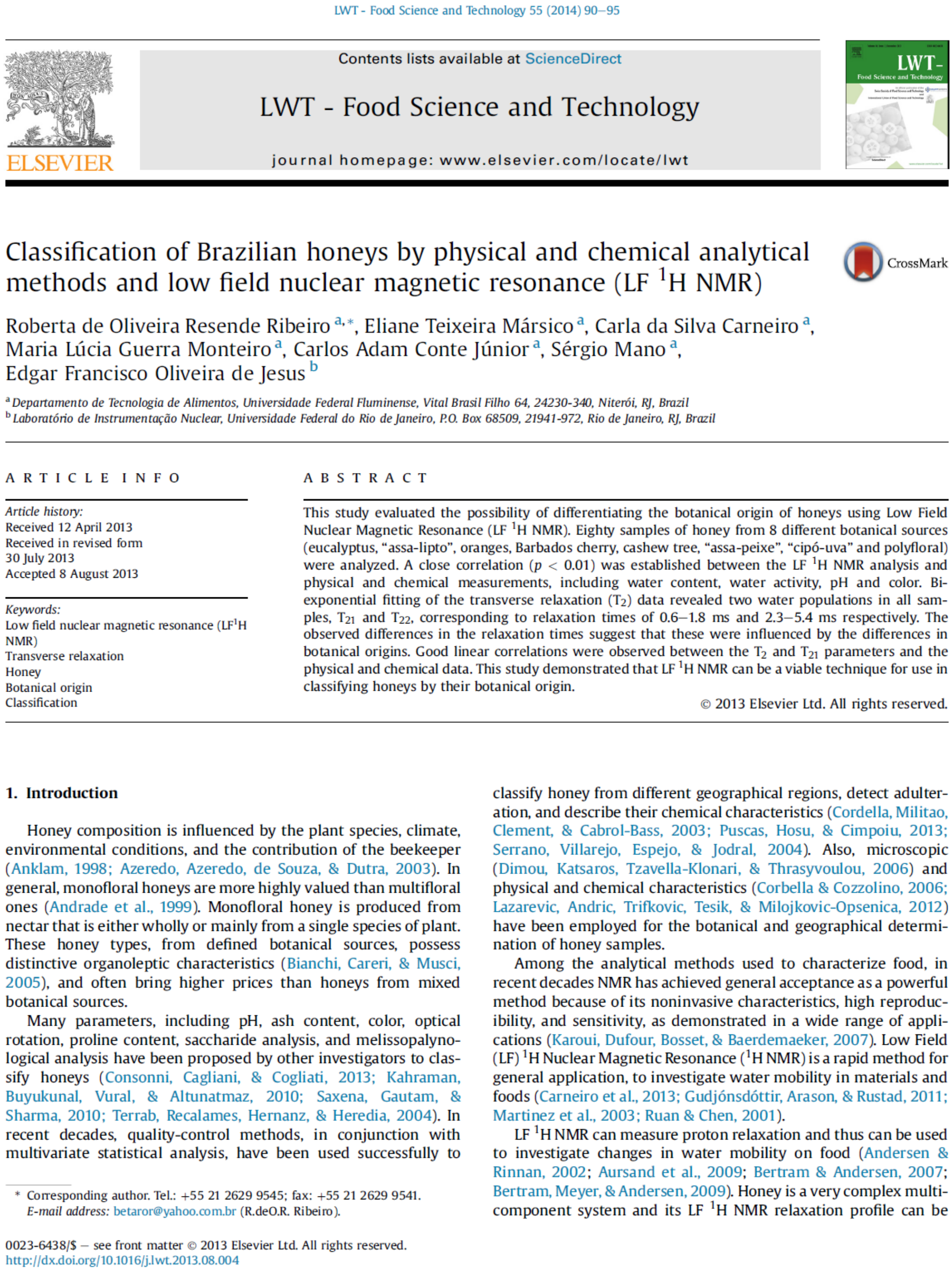
6 APÊNDICES

6.1 PAPER I



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Classification of Brazilian honeys by physical and chemical analytical methods and low field nuclear magnetic resonance (LF ^{1}H NMR)

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ABSTRACT

This study evaluated the possibility of differentiating the botanical origin of honeys using Low Field Nuclear Magnetic Resonance (LF ^{1}H NMR). Eighty samples of honey from 8 different botanical sources (eucalyptus, “assa-lipto”, oranges, Barbados cherry, cashew tree, “assa-peixe”, “cipó-uva” and polyfloral) were analyzed. A close correlation ($p < 0.01$) was established between the LF ^{1}H NMR analysis and physical and chemical measurements, including water content, water activity, pH and color. Bi-exponential fitting of the transverse relaxation (T_2) data revealed two water populations in all samples, T_{21} and T_{22}, corresponding to relaxation times of 0.6–1.8 ms and 2.3–5.4 ms respectively. The observed differences in the relaxation times suggest that these were influenced by the differences in botanical origins. Good linear correlations were observed between the T_2 and T_{21} parameters and the physical and chemical data. This study demonstrated that LF ^{1}H NMR can be a viable technique for use in classifying honeys by their botanical origin.

1. Introduction

Honey composition is influenced by the plant species, climate, environmental conditions, and the contribution of the beekeeper (Anklam, 1998; Azeredo, Azeredo, de Souza, & Dutra, 2003). In general, monofloral honeys are more highly valued than multifloral ones (Andrade et al., 1999). Monofloral honey is produced from nectar that is either wholly or mainly from a single species of plant. These honey types, from defined botanical sources, possess distinctive organoleptic characteristics (Bianchi, Careri, & Musci, 2005), and often bring higher prices than honeys from mixed botanical sources.

Many parameters, including pH, ash content, color, optical rotation, proline content, saccharide analysis, and melissopalynological analysis have been proposed by other investigators to classify honeys (Consonni, Cagliani, & Cogliati, 2013; Kahraman, Buyukunal, Vural, & Altunatmaz, 2010; Saxena, Gautam, & Sharma, 2010; Terrab, Recalames, Hernanz, & Heredia, 2004). In recent decades, quality-control methods, in conjunction with multivariate statistical analysis, have been used successfully to classify honey from different geographical regions, detect adulteration, and describe their chemical characteristics (Cordella, Militao, Clement, & Cabrol-Bass, 2003; Puscas, Hosu, & Cimpoiu, 2013; Serrano, Villarejo, Espejo, & Jodral, 2004). Also, microscopic (Dimou, Katsaros, Tzavella-Klonari, & Thrasyvoulou, 2006) and physical and chemical characteristics (Corbella & Cozzolino, 2006; Lazarevic, Andric, Trifkovic, Tesik, & Milojkovic-Opsenica, 2012) have been employed for the botanical and geographical determination of honey samples.

Among the analytical methods used to characterize food, in recent decades NMR has achieved general acceptance as a powerful method because of its noninvasive characteristics, high reproducibility, and sensitivity, as demonstrated in a wide range of applications (Karoui, Dufour, Bosset, & Baerdemaeker, 2007). Low Field (LF) ^{1}H Nuclear Magnetic Resonance (^{1}H NMR) is a rapid method for general application, to investigate water mobility in materials and foods (Carneiro et al., 2013; Gudjónsdóttir, Arason, & Rustad, 2011; Martinez et al., 2003; Ruan & Chen, 2001).

LF ^{1}H NMR can measure proton relaxation and thus can be used to investigate changes in water mobility on food (Andersen & Rinnan, 2002; Aursand et al., 2009; Bertram & Andersen, 2007; Bertram, Meyer, & Andersen, 2009). Honey is a very complex multi-component system and its LF ^{1}H NMR relaxation profile can be

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modeled as a linear combination of characteristic relaxation times from the measurable hydrogens present in their structure. In LF ^{1}H NMR studies, proton relaxation is described by the relaxation time constants T_1 (longitudinal) and T_2 (transverse), where T_2 relaxation decay in food is multiexponential, indicating the presence of different water populations or water "pools" in the foods matrixes (Bertram, Andersen, & Karlsson, 2001; Finch, Harmon, & Muller, 1971). However, we are aware of no published reports on the use of Low Field Nuclear Magnetic Resonance (LF ^{1}H NMR) to classify honey samples of different floral origins.

The aim of this research was to investigate changes in the distribution of water, by means of LF ^{1}H NMR relaxation time measurements associated with physic and chemical measurements of pH, water activity, color, and water, sugar, and ash contents that might enable the classify honey samples of different floral origins.

2. Materials and methods

2.1. Sample

Eighty samples of honey were obtained from beekeepers in different mountain areas in the states of Rio de Janeiro and Minas Gerais in southeastern Brazil. The samples consisted of 10 honeys from each of the following genera and species of plants: eucalyptus (*Eucalyptus* sp.), orange (*Citrus* sp.), Barbados cherry (*Malphigia* sp.), cashew tree (*Anacardium occidentale*), "assa-peixe" (*Vernonia* sp.), "assa-lipto", and "Cipó-Uva" (*Cissus rhombifolia*), and 10 polyfloral honeys.

All the honey samples collected were stored at room temperature (18–23 °C) from the time of acquisition to the spectral analysis (maximum 4 weeks after the beekeepers removed the honey from the hives). The honey samples were stored in the dark in screw-cap jars.

2.2. Physical and chemical measurements

All measurements were made in triplicate. Physical and chemical analyses were performed following international recommendations (AOAC, 1995).

Water content was determined with an Abbé-type refractometer. All measurements were performed at 20 °C, after waiting for 6 min to allow the sample to reach equilibrium. The refractive index of the honey sample was correlated using Chataway Charts.

The honey pH was measured with a pH meter (Digimed® Model DM-32, São Paulo, Brazil). The electrode was immersed in a suspension made by mixing 10 g of honey with 75 mL of distilled water.

The intensity of the color was measured with a Minolta type CR-400 colorimeter (Konika Minolta®, Tokyo, Japan) according to the manufacturer's instructions. The instrument records the values for L^* (lightness—intensity of white color), a^* (redness—intensity of red color), and b^* (yellowness—intensity of yellow color).

The water activity was measured with a Pawkit meter (Decagon Devices, Pullman, WA, USA). This measures the water activity of the sample, based on its equilibrium relative humidity (ERH). Approximately 1 g of each honey sample was used to determine the water activity (a_w).

2.3. Low field NMR measurements

For the NMR measurements, a bench-top NMR analyzer with a working frequency of 13 MHz was used (MARAN DXR 2, Oxford Instruments®, Osney Mead, Oxford, UK). The measurements were performed on 15 g samples at 25 ± 1 °C in NMR tubes (50 mm diameter). All samples were analyzed in triplicate. The transverse relaxation time (T2) was measured with a Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence (Carr & Purcell, 1954; Meiboom & Gill, 1958), with 10 scans, 256 points, 100 ms between scans, and 100 μs between pulses of 90° and 180°. The LF-NMR relaxation curve was fitted to a multi-exponential curve with the software RI-WINFIT (version 2.5, Oxford Instruments®). T2 distributions were obtained using the software WinDXP, version 1.8.1.0 from Resonance Instruments®, distributed by Oxford Instruments®.

2.4. Statistical analysis

A one-way analysis of variance with repeated measures was used to identify differences among the botanical origins of the honeys, for each relaxation time (T_2 and $T_{21,}$ T_{22}) and physical and chemical parameters. When a significant *F* was found, additional post-hoc tests with Bonferroni adjustment were performed. Pearson correlations were used to examine the relationship between each physical and chemical (pH, water activity, color, and water, ash, and sugar contents) and transverse relaxation parameter (T_2 and T_{21}, T_{22}) independently of botanical origin. Statistical significance was set at the 0.01 level of confidence. All analyses were performed with a commercially available statistical package (SPSS Inc., Version 17.0, Chicago, Illinois, USA).

3. Results and discussion

3.1. Physical and chemical results

Table 1 shows the means, standard deviations and ranges of the data obtained from the analysis of the different physical and chemical parameters (pH, water activity, color, and water, ash, and sugar contents).

Water content was influenced by the climatic conditions, the season of the year, and the degree of maturity of honeys (Conti, 2000; Finola, Lasagno, & Marioli, 2007; White, 1978). All samples had low water content, within the limit (20 g/100 g) allowed for natural honeys (Table 1). In one study, the water content of honeys of different origins showed varietal differences over a range from 16 to 20 g/100 g (Kayacier & Karaman, 2008). During storage, higher water content could lead to undesirable honey fermentation caused by the action of osmotolerant yeasts, resulting in the formation of ethanol and carbon dioxide. The ethanol can be further oxidized to acetic acid and water, producing a sour taste (Chirife, Zamora, & Motto, 2006). The mean water content of the samples ranged from 16.20 g/100 g for "cipó-uva" to 20.00 g/100 g for the cashew honey. The values obtained in this study for monofloral honeys were similar to those obtained by other investigators for Brazilian honeys (Abadio Finco, Moura, & Silva, 2010; Welke, Reginatto, Ferreira, Vicenzi, & Soares, 2008) of different origins.

The water and sugar contents of honey are closely correlated, as described by Conti (2000). In this study, the regression coefficient was $r = 0.87$ ($p < 0.01$).

The carbohydrate composition depends on several factors including the botanical and geographical origins of the plant from which the honeydew or nectar was collected, and the environment, climate, and processing and storage conditions (Marghitas et al., 2009; Ouchemoukh, Schweitzer, Bachir Bey, & Djoudad-Kadji, 2010). Glucose and fructose are the major constituents of honey. In this study, the percentage of reducing sugars in the samples ranged from 66.2 g/100 g to 80.1 g/100 g. Our findings were similar to those of Ouchemoukh, Louaileche, and Schweitzer (2007). The values found for total sugar content in this study ranged from 66.7 to 85.4 g/100 g. These levels are close to those obtained by Arruda, Marchini, Moreti, Otsuk, and Sodré (2005), Komatsu, Marchini, and Moreti (2002), Moreti, Sodré, Marchini, and Otsuk (2009), and

Table 1
Range of the physicochemical parameters (moisture, pH, ash, Aw and color) of analyzed honeys from different origins botanicals.

Types of honeys	Moisture [g/100 g]	pH	Ash contents [g/100 g]	Aw	Color		
					L^*	a^*	b^*
Cashew tree	19.76 ± 0.12^{a} (19.60–20.00)	4.13 ± 0.02^{a} (4.10–4.13)	0.42 ± 0.03^{a} (0.39–0.43)	0.67 ± 0.01^{a} (0.66–0.68)	43.51 ± 0.37^{a} (43.02–43.90)	27.16 ± 0.71^{a} (26.33–28.03)	36.30 ± 0.71^{a} (35.31–36.97)
Polyfloral	19.52 ± 0.13^{b} (19.40–19.80)	4.02 ± 0.03^{b} (4.02–4.10)	0.30 ± 0.02^{b} (0.28–0.31)	0.64 ± 0.02^{b} (0.62–0.65)	53.72 ± 0.44^{b} (53.32–54.33)	3.14 ± 0.16^{b} (2.91–3.26)	26.83 ± 0.98^{b} (25.44–27.52)
Barbados cherry	18.46 ± 0.16^{c} (18.20–18.60)	4.0 ± 0.02^{c} (3.96–4.05)	0.26 ± 0.01^{c} (0.25–0.27)	0.61 ± 0.01^{c} (0.60–0.62)	63.72 ± 0.44^{c} (63.32–64.33)	6.99 ± 0.44^{c} (6.51–7.58)	39.81 ± 0.71^{c} (38.59–40.96)
"Assa-Peixe"	18.28 ± 0.10^{d} (18.20–18.40)	3.94 ± 0.01^{d} (3.93–3.96)	0.19 ± 0.02^{d} (0.17–0.20)	0.60 ± 0.02^{d} (0.59–0.61)	66.07 ± 1.1^{d} (65.65–66.43)	10.44 ± 0.49^{d} (10.03–11.13)	43.19 ± 0.33^{d} (42.73–43.47)
"Assa-Lipto"	17.75 ± 0.14^{e} (17.60–18.00)	3.92 ± 0.02^{e} (3.90–3.94)	0.18 ± 0.01^{d} (0.17–0.19)	0.58 ± 0.1^{e} (0.57–0.58)	68.65 ± 1.02^{e} (67.24–69.63)	10.44 ± 0.18^{d} (10.28–10.69)	40.65 ± 0.29^{e} (40.28–40.99)
Eucalyptus	17.29 ± 0.10^{f} (17.20–17.40)	3.79 ± 0.02^{f} (3.78–3.81)	0.12 ± 0.03^{e} (0.39–0.43)	0.57 ± 0.3^{f} (0.55–0.59)	70.55 ± 0.29^{f} (70.14–70.78)	16.39 ± 0.3^{e} (15.45–16.95)	33.08 ± 0.13^{d} (32.93–33.25)
Oranges	16.44 ± 0.20^{g} (16.20–16.40)	3.40 ± 0.01^{g} (3.39–3.42)	0.09 ± 0.02^{f} (0.07–0.10)	0.55 ± 0.01^{g} (0.54–0.56)	75.22 ± 0.32^{g} (74.78–75.49)	11.06 ± 1.14^{f} (9.45–11.95)	40.31 ± 1.51^{ce} (38.23–48.78)
"Cipó-Uva"	16.49 ± 0.1^{g} (16.40–16.60)	2.99 ± 0.02^{h} (2.97.-3.02)	0.07 ± 0.01^{a} (0.06–0.07)	0.53 ± 0.02^{h} (0.51–0.54)	80.79 ± 0.57^{h} (79.98–81.21)	62.57 ± 0.62^{g} (61.90–63.40)	37.19 ± 0.25^{f} (36.87–37.47)

a, b, c. .. Different letters in a column indicate significant differences ($p < 0.01$) between treatments (ANOVA).
L^* (darkness), a^* (redness), and b^* (yellowness).
A_w (water activity).

Sodré et al. (2007), who found total sugar contents of 67.8–88.3 g/100 g for Brazilian honey samples of different floral origins.

The mean percentage of apparent sucrose was 3.2 g/100 g, with a range of 2.2–3.9 g/100 g. The level of sucrose differs according to the degree of maturity and origin of the nectar composing the honey. Cantarelli, Pellerano, Marchevsky, and Camiña (2008) reported mean sucrose content in honey samples of 4.05 g/100 g.

In general, honey is acidic in nature, irrespective of its geographical origin. The pH of honey can be influenced by the different sources of nectar, soil, or combination of plants for honey composition, by the action of glucose oxidase, the action of bacteria as the honey ripens, and also the amount of minerals present in the honey (Finola et al., 2007). Honey pH values are of great importance during storage, as they influence the texture, stability and shelf-life of honeys (Baroni et al., 2009; Downey, Hussey, Jelly, Walshe, & Martin, 2005; Terrab et al., 2004). Most bacteria grow in a neutral and mildly alkaline environment, while yeasts and molds are capable of growth in an acidic environment (pH = 4.0–4.5) (Conti, 2000). The pH values of our samples ranged from 2.98 for "cipó-uva" to 4.15 for cashew-tree honeys. These values fall within the range usually observed for other natural Brazilian honeys, between 3.10 and 4.05 (Azeredo et al., 2003). The pH value of honey is not directly related to free acidity, because of the buffering action of the various acids and minerals present.

The water activity (a_W) of the honey samples varied from 0.57 to 0.70 (Table 1). Our results are quite similar to those for other honeys, for which the a_W values ranged from 0.53 to 0.67 (Lazaridou, Biliaderis, Bacandritsos, & Sabatini, 2004). The water activity is an important factor in promoting stability, by preventing or limiting microbial growth. However, the osmotolerant yeasts are able to grow at a minimum water activity of 0.6 (Chirife et al., 2006).

The color characteristics are presented in Table 1 with the values for L^* (lightness—intensity of white color), a^* (redness—intensity of red color), and b^* (yellowness—intensity of yellow color). Honey samples having an L value > 50 are considered lighter, and samples having an L^* value ≤ 50 are dark. Based on this classification, the honey samples studied here were relatively light, with L^* values ranging from 43.02 to 81.21. The lighter honeys were predominant (87.50%); only the cashew honeys had L^* lower than 50. The a^* values ranged from 2.91 to 63.40 and b^* values ranged from 25.44 to 43.47. Thus it is apparent that all the darker samples had red and yellow components. The color of a honey is closely related to its chemical composition, and is presumed to result primarily from the presence of pigments (carotenoids, flavonoids, derivatives of tannins and polyphenols), which are also known to have antioxidant properties (Frankel, Robinson, & Berenbaum, 1998). The color values obtained were within the expected ranges for each of the honeys studied. The results of other studies that used L^*, a^* and b^* to measure color were similar to this study (Soria, González, Lorenzo, Martínez-Castro, & Sanz, 2004; Terrab, Dıez, & Heredia, 2003). These showed quite low mean values of lightness L^*, between 38 and 41, coinciding with their dark color.

The physical property of honey that best correlates with the total mineral content is color. This result is consistent with Anklam (1998), who reported that dark honeys have a higher mineral content than pale honeys. A predominance of light colors was also previously reported by Moreti, Sodré, and Marchini (2006) for the 346 honey samples collected in six states in Brazil.

The wide variability of honey composition is also reflected in the ash content (Baroni et al., 2009). Ash content is one of several parameters that have been associated with the botanical and geographical origins of honey samples. The ash content in honey is generally low, and depends on the nectar composition of the predominant plants in its composition (Al-Khalifa & Al-Arify, 1999). In this study, the ash content of the samples ranged from 0.07 g/100 g

Table 2
Range of the LF ^{1}H NMR parameters obtained in honeys according to the different botanical origins.

Types of honeys	T_{21} [ms]	T_{22} [ms]
Cashew tree	1.88 ± 0.10^a (1.65–2.06)	5.49 ± 0.12^a (5.15–5.68)
Polyfloral	1.58 ± 0.07^b (1.44–1.71)	4.91 ± 0.08^b (4.71–5.05)
Barbados cherry	1.36 ± 0.12^c (1.11–1.63)	3.53 ± 0.10^c (3.33–3.72)
“Assa-Peixe”	1.31 ± 0.07^c (1.16–1.42)	4.07 ± 0.10^c (3.89–4.24)
“Assa-Lipto”	1.07 ± 0.07^d (0.96–1.22)	3.43 ± 0.09^c (3.32–3.59)
Eucalyptus	1.09 ± 0.12^d (0.89–1.32)	2.95 ± 0.09^d (2.75–3.10)
Oranges	0.84 ± 0.05^e (0.73–0.94)	3.30 ± 0.12^c (3.07–3.50)
“Cipó-Uva”	0.64 ± 0.04^f (0.57–0.74)	2.43 ± 0.06^e (2.29–2.54)

a, b, c. .. Different letters in a column indicate significant differences ($p < 0.01$) within each treatment (ANOVA).

for “cipó-uva” to 0.42 g/100 g for the cashew-tree honeys. In comparison, other investigators reported the following ranges for natural honeys: 0.28 g/100 g (Kahraman et al., 2010), 0.03–0.21 g/100 g (Khan, Kaiser, Raza, & Rehman, 2006) and 0.08–0.39 g/100 g (Ahmed, Prabhu, Raghavan, & Ngadi, 2007). The ash percentage was higher in dark honeys such as cashew tree (Table 1).

3.2. Low field NMR results

No previous studies of the relaxation component with Low Field Nuclear Magnetic Resonance on honeys of different botanical origins are available.

Having established the physical and chemical properties of honeys from 8 different botanical sources, we measured the NMR proton relaxation of these same samples. Our purpose was to evaluate the possibility of a correlation between these data, which could be used to determine the botanical origin of honey. Different methods were used to analyze the T_2 relaxation data: continuous distributed NMR relaxation curves, biexponential fitting of T_2 relaxation curves, and comparison of NMR and physical and chemical data.

3.2.1. Continuous distributed NMR (T_2) relaxation curves

Comparison of the continuous distributed curves revealed visible differences in the distribution of water mobility among the different botanical sources. The cashew honeys, which had higher water activity, tended to exhibit a slightly broader T_2 distribution than did the others with lower water activity. This is likely due to the differences in nectar composition and water distribution caused by different flowers.

3.2.2. Bi-exponential fitting of T2 relaxation curves

Bi-exponential fitting of the transverse relaxation data resulted in the observation of two water populations in all samples, resulting in a faster relaxation time T_{21} in the range of 0.57–2.10 ms, and a slower relaxation time T_{22} in the range of 2.30–5.67 ms, depending on the botanical origin (Table 2).

In the chemometric analysis of the data, an ANOVA was carried out to ascertain those variables with mean values that were statistically different for each kind of honey, with a probability of 99% (Table 2). The results for the ANOVA carried out for relaxation time T_{21} showed that the mean contents of all honeys, with the exception of eucalyptus and “assa-lipto”, were significantly different. This indicated that the relaxation time T_{21} of honeys varies according to the botanical origin. On the other hand, the relaxation times T_{22} of all honey samples were statistically different ($p < 0.01$), which confirms the effectiveness of screening of honeys from different botanical origins. This model would allow us to save time in the determination of the floral origin of honey.

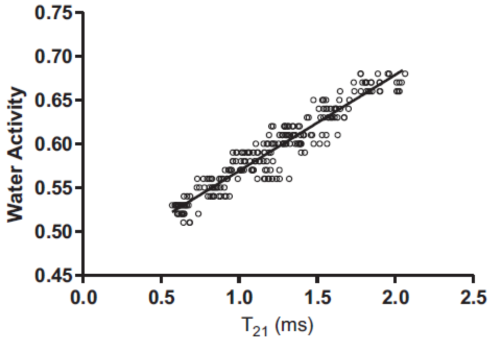

Fig. 1. Correlation between Transverse relaxation time (T_{21}) and water activity (A_w) in honeys, according to the different botanicals origins.

3.2.3. Comparison of NMR and physical and chemical data

The NMR data obtained by biexponential fitting were correlated with the physical and chemical parameters. Significant correlations were observed between the NMR relaxation parameters and the physical and chemical properties, with the exception of sugar content ($rT_{21} = -0.66$; $rT_{22} = -0.57$). The relaxation times T_{21} and T_{22} showed direct correlations with the water content ($rT_{21} = 0.92$; $rT_{22} = 0.77$), pH ($rT_{21} = 0.86$; $rT_{22} = 0.73$) and water activity ($rT_{21} = 0.91$; $rT_{22} = 0.83$).

In this study, the water activity and pH increases were associated with longer T_{21} relaxation times in honey. We attributed this to the composition of honeys from different botanical origins.

As seen in Fig. 1, water activity was correlated with T_{21} ($r = 0.91$) for all honey samples. According to Pelizer et al. (2003), the water activity influences microbial growth and enzymatic and biological processes. Each organism has a minimum water activity required to perform its metabolic activities. The optimum water activity for fungi is around 0.70, for yeast 0.80, and for bacteria 0.90.

Values of pH are dependent on the amount of ions in the honey, which is correlated with the total mineral content. Similar correlations were found by Vanhanen, Emmertz, and Savage (2011), between the total ash content of honey and the pH ($r = 0.86$). Bertram et al. (2001) also demonstrated that the relaxation times were efficient parameters for indicating changes in muscle pH. Although the change occurred in another matrix, this change in pH demonstrates the direct influences on the relaxation time.

The relaxation times (T_{21}) were correlated with the parameters L^* ($r = -0.91$), a^* ($r = -0.43$), and b^* ($r = -0.31$). The progressively lighter colors in the honeys and the increases in the relaxation time T_{21} were closely correlated with the decrease of the ash content ($r = 0.87$). The mineral content influences the color and taste of honey. The higher the content of metals and the darker the color, the stronger the honey will taste (Sancho, Muniategui, Huidobro, & Simal Lozano, 1991). González-Miret, Terrab, Hernanz, Fernández-Recamales, and Heredia (2005) demonstrated that the color of dark honeys (avocado, chestnut, honeydew, and heather) was closely correlated with the concentration of trace elements such as As, Cd, Fe, S, Pb and Ca. On the other hand, the colors of light and brown honeys (citrus, rosemary, lavender, thyme, and eucalyptus) were highly correlated with only Al and Mg.

We also observed a significant correlation between T_{21} and water ($r = 0.94$) content in honeys from different plants. Water content influences honey color, viscosity, flavor, density and refractive index, and is one of the most important physical and chemical parameters for the analysis of conservation and stability of foods in general (Mateo & Bosch-Reig, 1997; Sancho et al., 1991).

Estupinan and Sanjuan (1998) reported water contents of honey in the range of 13–25%, and stated that water content was affected by climate, season, and the water content of the original plant nectar.

4. Conclusions

The observations reported here open the way to the development of a tool for classification of honeys. Low Field ^{1}H Nuclear Magnetic Resonance (LF ^{1}H NMR) relaxation is a rapid noninvasive method to investigate the botanical origin of honey. The current reference methods are costly, require sophisticated technical training, and have a long acquisition time. Measurements made through LF^{1}H NMR with appropriate statistical analysis can be extended to accumulate a set of data for classification of the different botanical origins of honey samples, based on reliable clues.

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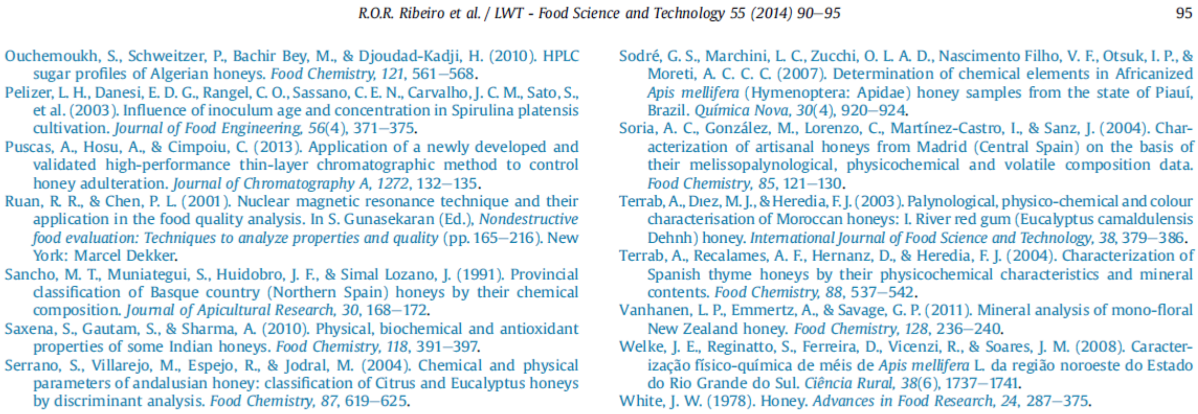

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6.2 PAPER II

INFLUENCE OF THE TIME/TEMPERATURE BINOMIAL ON THE HYDROXYMETHYLFURFURAL CONTENT OF FLORAL HONEYS SUBJECTED TO HEAT TREATMENT

Influência do binômio tempo e temperatura nos teores de hidroximetilfurfural em méis florais submetidos ao aquecimento

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Fernanda Lima Cunha[1], Carlos Adam Conte Junior[3], Sérgio Borges Mano[3]

ABSTRACT

Hydroxymethylfurfural (HMF) content is internationally recognized for its ability to indicate the freshness or lack of freshness of honey and can be used to judge the processing and storage condition of honey. The objective of the present study was to evaluate the evolution of HMF levels in fresh extracted honeys submitted to different temperatures ranging from 30 to 100° C, during pre-established time intervals (30, 45, 60, 180 and 720 minutes).The test was conducted in floral honey with an initial HMF content of 2.2 mg/Kg. The maximum value recommended by the Brazilian law (60 mg/Kg) was not exceeded in the samples subjected to heating for 30, 45 and 60 minutes regardless of the temperatures used. When the samples were heat treated during 180 minutes at 90° C the official value was surpassed. The highest values were observed in samples subjected to heating over 720 minutes, and the limit was exceeded at 70° C. The results obtained indicate that the HMF content gradually increases when the honey is heated at high temperatures for long periods. Therefore, we suggest a process optimization, considering the initial HMF content of the product and standardizing times and temperatures to ensure a good quality of the final product.

Index terms: Honey quality, HMF, heating.

RESUMO

O teor de hidroximetilfurfural (HMF) é reconhecido internacionalmente pela capacidade de indicar o frescor do mel, podendo ser usado para avaliar o processamento e condições de armazenamento do mel. Neste trabalho objetivou-se avaliar a evolução dos níveis de HMF em mel recém-obtido, submetidas a diferentes temperaturas que variaram de 30 a 100° C, por intervalos de tempo pré-definidos (30, 45, 60, 180 e 720 minutos). O ensaio foi realizado em mel floral, cujo valor inicial de HMF foi de 2,2 mg/Kg. O valor máximo preconizado pela legislação brasileira não foi ultrapassado nas amostras submetidas ao aquecimento por 30, 45 e 60 minutos independente das temperaturas. Por um período de 180 minutos, o limite legal de 60 mg/Kg foi extrapolado nas amostras tratadas a 90° C. Os valores mais elevados foram observados nas amostras submetidas ao aquecimento por 720 minutos, tendo o limite legal ultrapassado em temperatura mais branda (70° C). Com base nos resultados obtidos, pôde-se concluir que o conteúdo de HMF aumenta gradativamente quando o mel é exposto a altas temperaturas, por tempo prolongado. Sendo assim, sugere-se uma otimização nos processos de beneficiamento, considerando a quantidade inicial de HMF no produto, com processamento padronizado em temperaturas e tempo controlados para garantia da qualidade do produto final.

Termos para indexação: Qualidade do mel, HMF, aquecimento.

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INTRODUCTION

Honey is a natural product that must be delivered to the consumer with its essential composition and its quality minimally altered (CODEX, 1987). However, honey suffers a natural ageing process which starts on the field when it is harvested and placed in barrels by the beekeepers; it goes on with transport and storage previous to the industrial treatment and continues during the industrial process and even after it (SANCHO et al., 1992).

5-hydroxyimethylfurfural (HMF) is a furanic compound produced by sugar degradation (RAMIREZ et al., 2000), naturally formed as an intermediate in the Maillard Reaction (AMES, 1992) or from dehydration of hexoses in acid medium (BELITZ; GROSCH, 1999), mainly products with pH value up to 5.0 (DAMODARAN et al., 2010). The presence of simple sugars and water in acid medium favors the formation of this furanic compound (NOZAL et al., 2001).

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HMF formation process can be accelerated during the heat treatments applied to honey (KROH, 1994). However, in acid media, HMF can be formed even at low temperatures (LEE; NAGY, 1990), which explains the gradual increase of HMF levels during storage. Some factors, such as the use of metallic containers (WHITE, 1979), honey physico-chemical properties (pH, total acidity, mineral content), the floral origin (AANAM; DART, 1995) and the thermal stress to which the product is subjected in the hive (SPANO et al., 2006), directly influence HMF formation during storage. Due to the factors above mentioned, HMF is considered one of the degradation products more frequently used as honey quality indicator (TOSI et al., 2002; FALLICO et al., 2004), once this product is almost absent in fresh extracted honeys and its concentration increases with time (SPANO et al., 2006), inadequate exposure in retail outlets, aging and improper use of heat to make honey more liquid.

In recent decades, HMF has drawn the attention of the scientific community for its carcinogenic potential for humans. Some studies have shown that this metabolite can be converted *in vivo* to 5-sulfooxymethylfurfural (SMF), a genotoxic compound (SURH et al., 1994). In addition, at high concentrations, HMF is cytotoxic, causing irritation to eyes, upper respiratory tract, skin and mucous membranes (ULBRICHT et al., 1984; BRUCE et al., 1993). For this reason, the *Codex Alimentarius* and the European Commission have set a maximum HMF level for honey of 40 mg/Kg, except for honeys coming from tropical countries and honeys with low enzyme levels, the HMF limit of which was set in 80 and 15 mg/Kg respectively (CODEX, 1987; EUROPEAN COMMISSION, 2001). The Brazilian legislation recommends a maximum limit of 60 mg/Kg for all kinds of honey (BRASIL, 2000). The amount of HMF detectable in honey is directly related to the intensity of the heat and the exposure time applied during processing.

The objective of the present study was to evaluate the evolution of HMF content in fresh extracted honeys, subjected to different temperatures ranging from 30 to 100ºC, over pre-established time intervals (30, 45, 60, 180 and 720 minutes).

MATERIALS AND METHODS

Honey samples

Honey samples were directly obtained from an apiary located in the mountain region of Rio de Janeiro, in the municipality of Teresopolis, a region characterized by diverse vegetation with altitude tropical climate and average annual temperature of 16±2ºC. Approximately 6 Kg of multifloral honey were fractionated in 120 glass bottles with 20 mL capacity and stored at 20±1° C.

Treatments

In order to evaluate the effects of time and temperature on the variation of HMF levels in the samples of floral honey, heat treatments were performed, in triplicate, by immersion in a thermostatic bath with periodic shaking, set at the following temperatures: 30, 40, 50, 60, 70, 80, 90 and 100º C. During this stage, the samples were kept at those temperatures during 30, 45, 60, 180 and 720 minutes. Temperature was monitored with a thermocouple introduced at the geometrical center of the bottles. The time was measured from the moment the temperature reached the value established in the methodology. Then, the samples were cooled by immersion in cold water at 4±2º C and immediately analyzed.

Physico-chemical analysis

The samples of honey were submitted to physico-chemical analysis in order to verify their quality before undergoing heat treatment. The following techniques were used: determination of pH, fixed mineral residue, insoluble matter, reducing and non-reducing sugars, Lund reaction, acidity analysis, Fiehe reaction.

HMF content determination

The content of HMF in the samples was determined by Winkler spectrophotometry method. Five grams of honey were weighted, dissolved in distilled water and transferred to a 25mL volumetric flask and make up to the mark. Immediately after preparation a 2 mL aliquot was transferred to a test tube and 5mL of 10% p-toluidin solution in isopropanol and 1mL of 5% barbituric acid aqueous solution were added. In another test tube (reference) was added 2mL of honey solution, 5mL of p-toluidin solution and 1mL distilled water. The reagents were used in up to 2 minutes after preparation in order to ensure the stability of the solutions. The absorbance at 550 nm was determined using a spectrophotometer. The honey used contained an initial HMF value of 2.2 mg/Kg before heating.

Statistical Analysis

Data were statistically analyzed using the two-way analysis of variance (ANOVA), aiming at identifying differences in HMF content in honey caused by the interaction of time/temperature variables. Multiple regression was applied with HMF content as dependent variable and the sample immersion time in the hydrostatic bath and the temperature of the bath as independent variables. All the analyses were performed using a statistic package available in the market (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, California, USA).

RESULTS AND DISCUSSION

The values obtained during the experiment are presented in tables and figures that summarize the influence of the heat treatments on HMF content in the samples of floral honey analyzed. HMF content significantly varied ($P<0.001$) with the increase of temperature and immersion time to which the samples were subjected. The regression coefficient ($r=0.98$), indicated that only 2% of the regression variances do not depend on the variables studied. It was observed that HMF content increases slowly up to 70° C and, above this temperature, its production rate increases in a more significant way, mainly at temperatures above 80° C. The HMF content increase is more evident when the immersion time in the hydrostatic bath increases, reaching its highest value in the samples exposed to 100° C for 720 minutes, which presented an average value of 101.24 mg/Kg (Figure 1).

The HMF content variation was small in the treatments at 30, 40 and 50° C for up to 180 minutes, varying from 4.46 mg/Kg at 30° C during 30 minutes to 15.28 mg/Kg at 50° C for 180 minutes, indicating that the use of those temperatures in honeys with low initial HMF content does not cause a significant increase of this compound. In general, it can be observed that honeys heat treated at 40 and 50° C for up to 180 minutes does not present a significant difference of HMF formation ($P<0.05$). Karabournioti and Zervalaki (2001) reported a slightly significant increase of HMF content in orange honeys heat treated at 35° C, 45° C, 55° C and 65° C for 24 hours, from 2.25 mg/Kg (without heating) to 3.45, 3.75, 4.35 and 19.00 mg/Kg, respectively and concluded that mild temperatures up to 55° C, do not influence the increase of HMF regardless of the exposure time.

The effects of heating on HMF increase can be observed when the exposure time is longer, the time variable being the most significant factor in increasing HMF content, when temperatures between 30 and 90° C are considered. The results show that there was no significant difference ($p>0.05$) on HMF levels in samples submitted to temperatures of 30, 40, 50, 60, 70 and 80°C for 30 minutes. The values increased from 2.2 mg/Kg (fresh extracted honey) to 4.46, 5.68, 6.96, 7.12, 8.88 and 10.68 mg/Kg, respectively. Samples heated at 80, 90 and 100° C for 30 minutes, significantly differed ($P<0.05$) from samples kept in thermostatic bath for 45 minutes confirming the trend of increased levels of HMF when the heating time increases (Table 1).

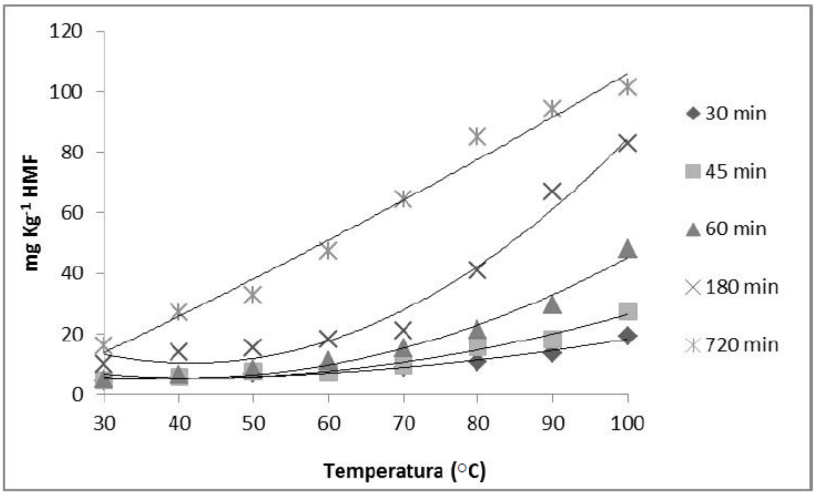

Figure 1 – Results of the determinations of HMF content according to temperature and time of immersion of honey samples.

Table 1 – Variation of the concentration of Hydroxyimethylfurfural (mg/Kg) in honey samples as a function of temperature and time of immersion in a thermostatic bath.

Temperature	Immersion time				
	30 min	45 min	60 min	180 min	720 min
30ºC	4.46 ± 0.15^{Aa}	4.77 ± 0.12^{Aa}	5.04 ± 0.90^{Aa}	9.97 ± 0.43^{Ba}	16.18 ± 0.37^{Ca}
40ºC	5.68 ± 0.83^{Aa}	5.82 ± 0.32^{Aa}	6.58 ± 0.53^{Aa}	14.05 ± 0.68^{Bb}	27.15 ± 0.18^{Cb}
50ºC	6.96 ± 0.13^{Aa}	7.39 ± 0.22^{Aa}	8.18 ± 0.14^{Aa}	15.28 ± 0.80^{Bb}	32.63 ± 0.37^{Cc}
60ºC	7.12 ± 0.22^{Aa}	7.33 ± 0.16^{Aa}	10.97 ± 0.10^{Aa}	18.32 ± 0.29^{Bb}	47.19 ± 3.19^{Cd}
70ºC	8.88 ± 0.37^{Aa}	9.61 ± 0.43^{Aa}	15.24 ± 0.19^{Bb}	20.96 ± 0.11^{Cb}	64.37 ± 0.11^{De}
80ºC	10.68 ± 0.28^{Ab}	15.62 ± 0.24^{Bb}	21.35 ± 0.30^{Cc}	41.03 ± 0.08^{Dc}	85.10 ± 0.26^{Ef}
90ºC	13.54 ± 1.10^{Ac}	18.36 ± 0.24^{Bc}	29.55 ± 0.30^{Cd}	66.96 ± 0.28^{Dd}	94.23 ± 0.56^{Eg}
100ºC	19.31 ± 2.40^{Ad}	27.48 ± 0.32^{Bd}	48.19 ± 1.35^{Ce}	82.79 ± 3.23^{De}	101.24 ± 0.35^{Eh}

The appearance of honey plays an important role on its commercial acceptance, once consumers demand a fluid, non-crystallized product. Fresh extracted honey is liquid. However, it may crystallize during storage at higher or lower speed depending on several factors such as origin (botanical and geographical), temperature, moisture content and sugar content (PIRO et al. 1996; BARTH; SINGH,1999). In order to delay the natural crystallization process and ensure stability during its shelf-life, fresh honey is usually submitted to heating before being packed, with the purpose of dissolving sugars and destroying yeasts (BATH; SINGH, 1999; TOSI et al., 2002). The results of the present study indicate that honey should be heated at mild temperatures, between 40º C and 50º C, heat treatments above 90º C are not recommended.

According to Jeanne (1985) de-crystallization of finely crystallized honeys requires different times and temperatures, for example, it is recommended that a 20 Kg recipient is heated at 40º C during 24 hours, and the author suggests a time/temperature binomial of 72 h at 50º C for a 300 Kg recipient. However, the present study showed that high contents of HMF (2.15 and 32.63 mg/Kg, for samples exposed to 40 and 50º C, for 12 hours, respectively), can be obtained when heat treatments are applied during long periods. Although the maximum level of HMF was not exceeded at temperatures up to 50º C, the value was close to that recommended by international legislation, which could limit the international trade of samples submitted to heating. An effective temperature control should be kept during honey de-crystallization stages, because a considerable HMF increase may occur when heating at 70º C as shown in the present study. After 720 minutes heating, the HMF content was 64.37 mg/Kg, a value that exceeds the value allowed by the Brazilian law (60mg/Kg) (BRASIL, 2000). Fallico et al. (2004), observed a significant increase of HMF content in eucalyptus honey subjected to 70º C, for 96 hours, from an initial value below detection level to 513 mg/Kg, in agreement with data obtained in the present study.

At high temperatures, such as 80 and 90º C, a gradual increase was observed that significantly varied (P<0.01) with heating time, which was the factor that mostly influenced the increase of HMF content at these temperatures of samples that when heated at 90º C presented HMF levels of 29.55 and 66.96 mg/Kg, after 60 and 180 minutes heat treatment, respectively. However, Turhan et al. (2008) presented different results. They reported low HMF contents in floral honeys subjected to 75 and 90º C heat treatments for up to 90 minutes. Those authors concluded that there was no significant increase of HMF in floral honeys heated at 90º C for up to 90 minutes, obtaining a maximum HMF content of only 11.24 mg/Kg.

It should be enhanced that, at 100º C, the formation of HMF presented a different behavior than at other temperatures, and, in this case, temperature was the factor that most significantly (P<0.01) influenced the increase of HMF, since there was an increase at all the times studied. In the same way, Turhan et al. (2008), described an increase of HMF content in floral honeys heated at 100º C for 30, 45, 60, 75 and 90 minutes, from 0.62 mg/Kg in samples without heating to 13.99, 26.4, 37.98, 55.41 and 73.78 mg/Kg, respectively. These authors observed a low HMF content in honeys subjected to heat treatments up to 90º C, demonstrating that extreme heating significantly accelerates HMF formation.

Tosi et al. (2002) studied the effects of applying temperatures between 100 and 160º C in short time intervals, varying from 14 to 60 s. The authors concluded that temperatures above 140º C, even when applied during short time intervals, considerably increased HMF contents, which reached values above those recommended by international standards, demonstrating the influence of temperature on the increase of HMF content even in short time intervals. In the present study, we observed an increase of HMF content in samples submitted to heat treatment at 100º C, regardless the heating time, significantly differing ($P<0.05$) at all levels, reinforcing the relevance of the study and in accordance with the above mentioned authors. After 60 minutes heating, HMF concentration was 48.19 mg/Kg, exceeding the limits recommended by the European legislation. Such increasing trend was maintained up to 720 minutes, when HMF concentration reached the highest levels, above100 mg/Kg.

The maximum value recommended by the Brazilian legislation of 60 mg/Kg (BRASIL, 2000), was not exceeded in the samples subjected to heating for 30, 45 and 60 minutes, regardless the temperature used. However, increasing the time that the samples were kept in the thermostatic bath to 180 minutes, the samples exceeded the legal values at temperatures above 80º C. It was also observed that samples heated for 180 minutes significantly differed ($P<0.05$) of samples heated for 720 minutes regardless the temperature used.

The samples heated during a 720- minute period did not exceed the acceptable limit of 60 mg/Kg, when the temperature was 70º C and, the samples submitted to 40º C, presented an average HMF content of 27.15 mg/Kg. Some authors observed that the increase of HMF is not related only to heating, and can occur during long storage periods, regardless the temperature. Khalil et al. (2010) studied HMF concentration in Malasian honeys stored during more than a year. They concluded that honey samples when stored during 12 to 24 months presented HMF concentrations that exceeded the recommended levels, reaching values of 118.47 and 1139.95 mg/Kg, respectively. Kalábová et al. (2003) demonstrated that the content of HMF gradually increases during storage, eventually reaching values above those recommended by current legislation. Therefore, the time/temperature binomial used for honey processing should be as low as possible, considering that HMF increase may be influenced by factors other than exposure to adverse temperatures.

However, it is important to emphasize, that, in this study, honey was collected at Teresopolis municipality, a region characterized by altitude tropical climate, with cold, dry winters and template, humid summers, with annual average temperature of 16±2º C and relative humidity of the air of 84%. Although the European legislation is more flexible with products from tropical regions, allowing contents of up to 80 mg/Kg, the floral honey acquired for this study was not exposed to high temperatures in the hive, which explains the initial low content of HMF (2.2mg/Kg). Thus, we used 40 mg/Kg and 60 mg/Kg, as references, the first is the value recommended by the Codex Alimentarius (CODEX, 1987) and the European Union Council (EUROPEAN COMMISSION, 2001) for honeys of non-tropical regions and the second the value recommended by the Brazilian legislation (BRASIL, 2000). These values are represented in figure 1, by grey and black horizontal lines.

CONCLUSION

HMF content significantly increases with the exposure to high temperatures during a long time. Considering the positive correlation between the thermal treatment and the increase of HMF content in fresh extracted honeys, it is suggested that HMF content is used as an indicator of honey quality loss caused by heating. Honeys with low initial HMF content may be submitted to temperatures up to 90ºC for periods up to 60 minutes, for de-crystallization. However, for longer periods of time, up to 180 minutes, the temperature should not exceed 70º C. Since honey overheating is not necessary for the filling process, mild temperatures should be used during limited times to reduce viscosity and prevent crystallization, without causing a significant increase of HMF content.

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6.4 FIGURAS RELATIVAS À ETAPA EXPERIMENTAL

6.4.1 Méis de diferentes origens florais

Fig. 1. Separação das amostras de méis de diferentes origens florais para posterior análise de LF ^{1}H NMR (Fonte: Arquivo pessoal)

6.4.2 Méis adulterados com High Fructose Corn Syrup

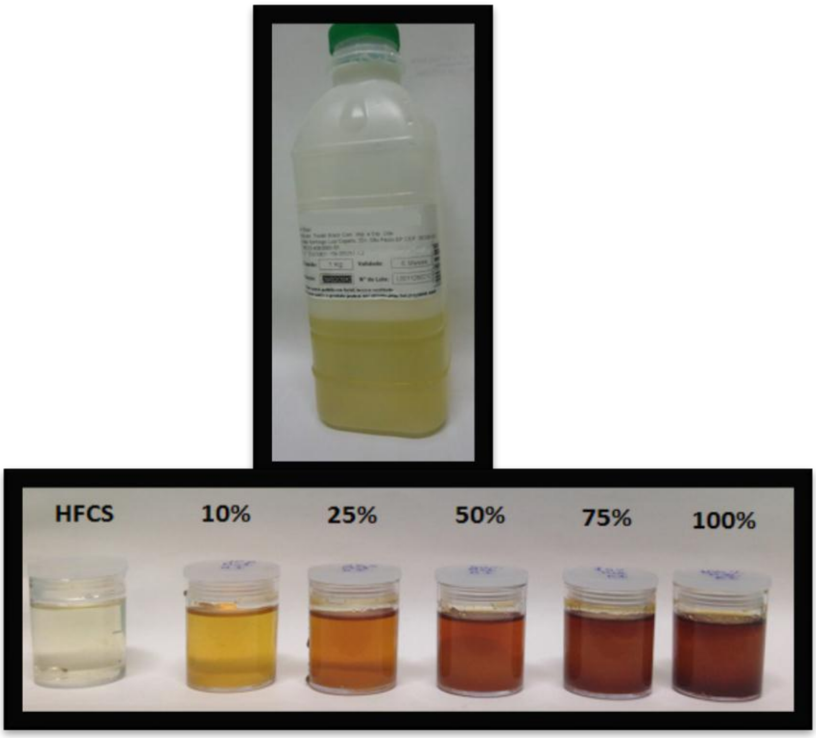

Fig. 1. Separação das amostras de méis fraudados com diferentes porcentagens de High Fructose Corn Syrup (HFCS).

6.4.3 Equipamento de LF ^{1}H NMR

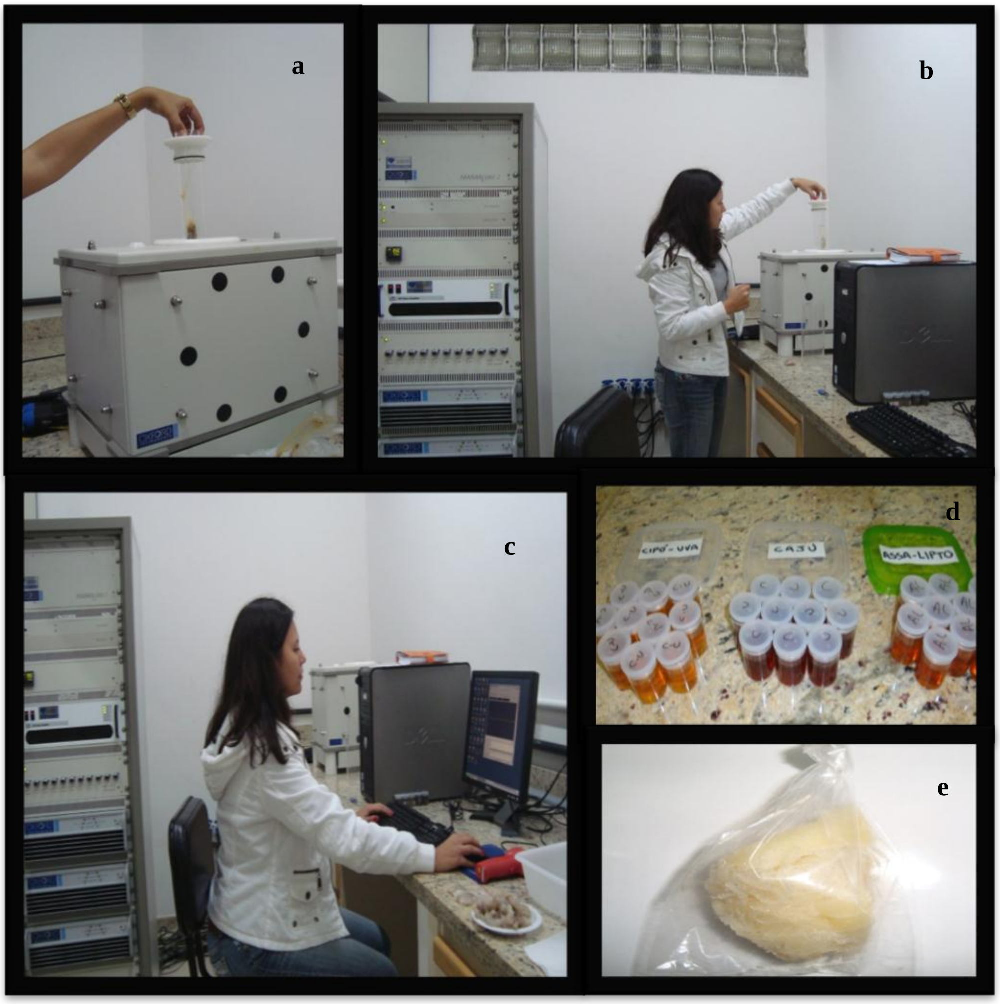

Fig (1a) Cilindro de vidro para colocação das amostras em equipamento de RMN, MARAN DXR 2$^{®}$ (Oxford Instruments); (1b e 1c) Equipamento de RMN, MARAN DXR 2$^{®}$ (Oxford Instruments); (1d) Amostras de méis de diferentes origens florais; (1e) Amostras de bacalhau do atlântico (*Gadus morhua*).