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ALIMENTOS**

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**Produção de microcápsulas de norbixina por *spray-drying*: avaliação da
estabilidade e aplicação em bebidas isotônicas como corante natural**

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Co-orientadora: Prof^a. Dr^a. Simone Hickmann Flôres**

**PORTO ALEGRE
Fevereiro, 2017**

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Produção de microcápsulas de norbixina por *spray-drying*: avaliação da estabilidade e aplicação em bebidas isotônicas como corante natural

Dissertação apresentada ao Programa de Pós-graduação em Ciência e Tecnologia de Alimentos da Universidade Federal do Rio Grande do Sul, como um dos requisitos para a obtenção do grau de Mestre em Ciência e Tecnologia de Alimentos.

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“He aprendido que el mundo
quiere vivir en la cima de la
montaña, sin saber que la
verdadera felicidad está en la
forma de subir la escarpada”

Gabriel García Márquez

RESUMO

A cor dos alimentos é um dos principais fatores que influenciam na preferência dos consumidores. Os corantes naturais além da coloração podem oferecer benefícios à saúde humana. A norbixina é um carotenoide que pode ser produzido a partir da saponificação de bixina presente nas sementes de urucum. Tal composto apresenta propriedades funcionais que estão relacionadas a promoção de uma vida saudável, pois atua como protetor celular, é comumente usado como corante natural em produtos processados, entretanto, sua estrutura química torna-o susceptível à degradação por fatores ambientais como oxigênio, luz e alta temperatura. A microencapsulação é uma alternativa para melhorar a estabilidade e solubilidade deste carotenoide. Neste trabalho foram encapsulados cristais de norbixina com 100% de pureza (comprimento de onda de 453 nm) usando a técnica de secagem por atomização (spray drying). Foram utilizados goma arábica (GA) e maltodextrina (MD) em diferentes proporções como materiais de parede. As diferentes formulações (MD:GA 100:0; 85:15; 65:35; 50:50; 35:65; 15:85; 0:100) foram preparadas com a mesma quantidade de núcleo (norbixina) e percentual de sólidos solúveis totais. A formulação com 100% GA mostrou a maior eficiência de microencapsulamento (74,91% - 226,40 µg/g) e foi avaliada a sua atividade antioxidante da pelo método ABTS ($77,77 \pm 0,59$ µmol TE/g microcápsula), verificou-se que a norbixina mantém a sua atividade antioxidante depois do processo de microencapsulamento. O estudo de estabilidade das microcápsulas de norbixina (MCN) foi conduzido em sistema modelo aquoso a temperaturas de 60, 90 e 98°C por 300 min. A cinética de degradação seguiu uma reação de primeira-ordem. A energia de ativação (E_a) requerida para degradação foi de 15,08 kcal/mol, o dobro da E_a requerida para a norbixina livre. As MCN mostraram uma alta estabilidade térmica. Finalmente, as MCN foram aplicadas em bebidas isotônicas sabor tangerina. Utilizando os parâmetros de cor do sistema CIELab foi possível obter a coloração laranja com uma baixa concentração de norbixina ($2,86 \pm 0,02$ µg norbixina/mL). A bebida isotônica (BIT) adicionada de MCN mostrou estabilidade sob condições aceleradas de armazenamento (luz e aquecimento), pois os resultados indicaram um maior tempo de vida média (29,71 dias) em comparação com a BIT adicionada de norbixina não encapsulada (6,56 horas). De acordo com essa pesquisa, os dados obtidos indicaram a potencialidade da utilização da microencapsulação para aumentar a estabilidade da norbixina e assim obter um corante natural com efetiva aplicação em matrizes modelo aquoso (bebidas).

Palavras-chave: urucum; corante natural; goma arábica; maltodextrina; carotenoides; estabilidade; norbixina; microencapsulação; bebida isotônica

ABSTRACT

Color is one of the main attributes in processed food that influences their preference and acceptance directly from consumers. Besides, of their ability of coloration, the natural dyes can offer benefits in human health. The norbixin is a carotenoid that can be produced from saponification of bixin present in annatto seeds. This compound shows functional properties that are related to the promotion of a healthy life, since it acts as cellular protectors, and is commonly used as a natural dye in processed food, however, its chemical structure makes it susceptible to degradation by environmental factors such as light, oxygen, and high temperature. Microencapsulation is used to improve the stability and solubility of the carotenoid. In this study, were encapsulated crystals of norbixin with 100% of purity (wavelength of 453 nm) by spray drying. Gum arabic and maltodextrin were used in different proportions as wall materials. The different formulations (MD:GA 100:0; 85:15; 65:35; 50:50; 35:65; 15:85; 0:100) were prepared with the same quantity of core (norbixin) and total solids soluble percentage. The formulation with 100% of arabic gum shows a high microencapsulation efficiency (74.91% - 226.40 $\mu\text{g/g}$ of microcapsules) and was evaluated its antioxidant activity with ABTS assay ($77.77 \pm 0.59 \mu\text{mol TE/g}$ microcapsules), was verified that norbixin keep its antioxidant activity after microencapsulation process. The stability study of norbixin microcapsules (MCN) was carried out in aqueous model system at temperatures of 60, 90 and 98°C for 300 min. Thermal degradation kinetics in aqueous model systems followed a first order kinetic reaction. The activation energy (E_a) required for degradation was $E_a = 15.08 \text{ kcal/mol}$, double than required for free norbixin. MCN showed a high thermal stability with longer shelf life. Finally, the MCN were applied in isotonic tangerine soft drinks without exceeding the use of food additives regulations. Based on the parameter of CIELab system was possible to get an orange tonality by using a lower concentration of norbixin ($2.86 \pm 0.02 \mu\text{g norbixin/mL}$). The isotonic beverage (BIT) added of MCN shows a stability during storage on accelerated conditions (heat and light) since the results indicated a high half-life time (29.71 days) when was compared with an BIT added of norbixin non-encapsulated (6.56 hours). According to this research, the results obtained showed the potential of the use of microencapsulation to increase the stability of norbixin, thus obtaining a natural dye with an effective application in aqueous matrix, mainly in beverages.

Keywords: annatto; natural dye; arabic gum; maltodextrin; stability; carotenoids; norbixin; microencapsulation; isotonic beverage

LISTA DE FIGURAS

Figura 1. Frutos e sementes de urucum (<i>Bixa orellana</i> L.)	14
Figura 2. Estrutura da bixina nas formas <i>cis</i> - e <i>trans</i>	15
Figura 3. Estrutura da norbixina nas formas <i>cis</i> - e <i>trans</i>	16
Figura 4. Esquema da degradação de <i>cis</i> -bixina.....	20
Figura 5. Imagens do processo para a obtenção dos cristais de bixina	29
Figura 6. Imagens do processo para a obtenção dos cristais de norbixina	30
Figura 7. Curva padrão de norbixina.....	31
Figura 8. Processo de microencapsulamento	32
Figura 9. Avaliação da umidade das microcápsulas de norbixina	32
Figura 10. Extração da norbixina para avaliar eficiência de microencapsulamento ...	34
Figura 11. Avaliação da cor das microcápsulas de norbixina	36
Figura 12. Avaliação da estabilidade térmica das microcápsulas de norbixina	38
Figura 13. Bebidas isotônicas de tangerina: a) adicionada de microcápsulas de norbixina; b) bebida comercial; c) adicionada de cristais de norbixina	39
Figura 14. Armazenamento de amostras de bebidas isotônicas de tangerina adicionadas de microcápsulas e cristais de norbixina sob condições aceleradas (luz e calor)	41
Figura 15. Amostras de bebida isotônica de tangerina adicionada de microcápsulas de norbixina ao início e fim do experimento.....	42

LISTA DE TABELAS

Tabela 1. Métodos de extração de corantes de urucum.	17
Tabela 2. Teores de pigmento nos corantes de urucum segundo o método de extração.....	19
Tabela 3. Principais formas e aplicações dos corantes do urucum.....	26

SUMÁRIO

INTRODUÇÃO	10
CAPITULO 1:	
REVISÃO BIBLIOGRÁFICA	14
1.1 Planta de urucum	14
1.2 Carotenoides nas sementes de urucum.....	15
1.2.1 <i>Bixina</i>	15
1.2.2 <i>Norbixina</i>	16
1.2.3 <i>Métodos de extração dos pigmentos de urucum</i>	16
1.3 Corante de urucum comercial.....	18
1.4 Estabilidade.....	19
1.4.1 <i>Estabilidade térmica</i>	19
1.4.2 <i>Ação antioxidante</i>	21
1.5 Encapsulamento.....	23
1.5.1 <i>Agentes de encapsulação</i>	24
1.5.2 <i>Processos de encapsulamento na indústria de alimentos</i>	25
1.6 Aplicações do urucum na indústria alimentícia	26
CAPITULO 2:	
MATERIAL E MÉTODOS	28
2.1 Isolamento do padrão de bixina	28
2.2 Isolamento do padrão de norbixina.....	29
2.3 Análise de espectrofotometria UV/Visível	30
2.4 Microencapsulação dos cristais de norbixina.....	31
2.4.1 <i>Rendimento do processo</i>	32
2.4.2 <i>Teor de umidade</i>	33
2.4.3 <i>Eficiência de Microencapsulação (EM)</i>	33
2.4.4 <i>Atividade antioxidante método ABTS</i>	34
2.4.5 <i>Solubilidade em água</i>	35
2.4.6 <i>Análises de colorimetria</i>	35
2.4.7 <i>Distribuição de tamanho de partícula</i>	36
2.4.8 <i>Microscopia eletrônica de varredura (MEV)</i>	36
2.4.9 <i>Cromatografia Líquida de Alta Eficiência (CLAE)</i>	37
2.5 Avaliação da estabilidade térmica das microcápsulas	37
2.6 Aplicação das microcápsulas obtidas: corante natural em bebidas isotônicas de tangerina	38

2.6.1	<i>Preparação de uma bebida isotônica de tangerina (BIT)</i>	39
2.6.2	<i>Acidez, pH e sólidos solúveis totais (°Brix)</i>	40
2.6.3	<i>Cor</i>	40
2.6.4	<i>Estabilidade das bebidas sob condições aceleradas de armazenamento</i>	40
2.7	<i>Análise Estatística</i>	42

CAPITULO 3:

Artigo 1:	Microencapsulation efficiency of norbixin encapsulated by spray-drying using gum arabic and maltodextrin as wall materials.....	44
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Artigo 2:	Thermal stability of norbixin microcapsules obtained by spray-drying.....	67
------------------	---	----

Artigo 3:	Stability assessment of isotonic tangerine soft drink added of norbixin microcapsules obtained by spray drying as a natural dye	84
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CAPITULO 4:

DISCUSSÃO GERAL	107
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CONCLUSÕES	109
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REFERÊNCIAS	110
--------------------------	-----

INTRODUÇÃO

A cor é considerada o principal atributo organoléptico que influencia a aceitação e preferência de um alimento. O uso de aditivos para tornar os alimentos e outros produtos visualmente mais atrativos para os consumidores é muito comum nas indústrias de alimentos e química. Por sua vez, a crescente demanda por corantes naturais é justificada pela sua toxicidade mínima ou inexistente (RODRIGUES *et al.*, 2014). Neste contexto os carotenoides são classe de compostos que apresentam poder corante e têm sido amplamente utilizados para este propósito na indústria de alimentos (RIOS E MERCADANTE, 2004).

O pigmento natural de urucum é o carotenoide mais utilizado na indústria brasileira e representa cerca de 90% dos corantes naturais utilizados nacionalmente e 70% em nível mundial (BARBOSA *et al.*, 2005; SOUSDALEFF *et al.*, 2013). Este pigmento é obtido das sementes dos frutos do urucuzeiro (*Bixa orellana* L.), uma árvore de clima tropical nativa da América do Sul e outras regiões tropicais da América Central, África e Ásia (LORENZI, 2000). Os pigmentos presentes são carotenoides e compreendem uma grande quantidade de *cis*-bixina e outros componentes minoritários, tais como *trans*-bixina, *cis-trans*-norbixina e norbixina (RODRIGUES *et al.*, 2014).

Na forma de corante natural, os extratos de urucum são usados em manteiga, queijo, produtos de panificação, óleos, sorvetes, salsichas, cereais e produtos extrusados, molhos para saladas, cereais matinais, refrigerantes e licores, e são relativamente baratos, quando comparados com outros pigmentos naturais (CARDARELLI *et al.*, 2008).

No entanto, os carotenoides têm um sistema de ligações duplas conjugadas que podem sofrer alterações quando são adicionados em um alimento e apresentam, como consequência, degradação e perda da cor. Isto os torna susceptíveis a altas temperaturas, disponibilidade de oxigênio, luz e pH baixo, com redução de sua estabilidade e limitação de sua utilização em alguns produtos alimentares (RIOS E MERCADANTE, 2004).

A crescente demanda para o uso de corantes naturais, como um substituto para a coloração sintética, tem estimulado o desenvolvimento de novas pesquisas nas formas de conservação desses pigmentos (AZEREDO, 2005), com destaque para a

microencapsulação e a adição de antioxidantes nas formulações (VALDUGA *et al.*, 2008).

A microencapsulação de corantes tem como objetivo protegê-los contra a oxidação, com aumento da vida de prateleira, além de permitir uma solubilização mais eficiente e melhor incorporação em alimentos (FAVARO-TRINDADE *et al.*, 2008). Existem várias técnicas que podem ser utilizadas para a microencapsulação de ingredientes alimentares, como a extrusão, leiteo fluidizado, liofilização, secagem por atomização, inclusão molecular e a inclusão em lipossomas (AZEREDO, 2005).

A técnica de secagem por atomização (*spray drying*) é a técnica mais utilizada na indústria de alimentos em razão da sua eficácia, fácil disponibilidade de equipamentos e baixos custos de produção, especialmente quando comparado com a maioria das outras técnicas (BARROS E STRINGHETA, 2006). A técnica envolve a atomização de emulsões em um meio de secagem a alta temperatura, o que causa uma evaporação de água muito rápida, o que por sua vez resulta na formação de uma cápsula e o aprisionamento do ingrediente dentro de um material de revestimento (FAVARO-TRINDADE *et al.*, 2008).

Cabe ressaltar que um dos principais fatores que influenciam a estabilidade dos compostos encapsulados é a natureza do material de encapsulação. O agente de encapsulação ideal deve mostrar propriedades emulsificantes, ter a capacidade para formar películas, possuir biodegradabilidade, ter baixa viscosidade e baixa higroscopicidade, e deve também apresentar um baixo custo. Na prática, é raro que só um agente de encapsulação possa ter todas as propriedades mencionadas, tornando-o comum a utilização de uma combinação de dois ou mais componentes (AZEREDO, 2005).

A goma arábica é o agente de encapsulação mais comumente usado na secagem por atomização, apresenta uma alta solubilidade em água e uma baixa viscosidade na solução, que representam duas características que a torna um ingrediente atrativo. A goma arábica já foi utilizada para microencapsular bixina através de secagem por pulverização, contudo a sua capacidade para formar complexos com norbixina hidrossolúvel ainda não foi estudada (GUAN E ZHONG, 2014).

Por sua vez, a maltodextrina têm baixa capacidade de emulsificação e baixa viscosidade em concentrações muito elevadas e têm sido estudada como possível

substituto para goma arábica em emulsões atomizadas ou em conjunto com a goma arábica para satisfazer as propriedades requeridas pelo material de encapsulação (AZEREDO, 2005).

A produção de pigmentos naturais microencapsulados podem oferecer uma grande vantagem para a indústria de alimentos uma vez que podem apresentar potencialidade para sua aplicação como aditivos naturais em diversas matrizes alimentares (sólidas ou líquidas) visando a substituição de corantes sintéticos e fornecendo um produto diferenciado e mais atrativo para o consumidor.

O composto encapsulado poderia ser mais solúvel em sistemas modelo aquoso que facilitaria sua aplicação em bebidas como corante natural, além de ser mais estável em condições drásticas de processamento ou armazenamento (luz e calor), tendo como resultado um produto final uma maior vida de prateleira.

OBJETIVOS

O objetivo geral deste trabalho foi elaborar, caracterizar, e avaliar a estabilidade de microcápsulas de norbixina extraída de sementes de urucum, além de avaliar sua aplicação em bebida isotônica para serem utilizadas como corante natural.

Dentro desse contexto os objetivos específicos deste trabalho foram:

1. Realizar a extração e cristalização da norbixina de sementes de urucum;
2. Realizar a microencapsulação dos cristais de norbixina obtidos com agentes de encapsulação (maltodextrina e goma arábica) em diferentes combinações;
3. Avaliar o rendimento e eficiência do processo de microencapsulação realizada com os diferentes agentes de encapsulação;
4. Caracterizar as microcápsulas produzidas quanto a sua umidade, concentração de norbixina, solubilidade, morfologia, cor, e atividade antioxidante;
5. Avaliar a estabilidade térmica em sistemas-modelo aquosos das microcápsulas de norbixina que apresentaram a maior eficiência de microencapsulação;
6. Realizar a aplicação das microcápsulas de norbixina em sistemas-modelo aquosos (bebidas isotônicas de tangerina) como corante natural;
7. Avaliar a estabilidade da norbixina nas bebidas isotônicas durante o armazenamento sobre condições aceleradas de luz e calor;

8. Comparar os efeitos das condições aceleradas de armazenamento na estabilidade de bebidas isotônicas aplicadas com microcápsulas ou cristais de norbixina.

Este trabalho está organizado na forma de capítulos. O **Capítulo 1** apresenta a fundamentação teórica dos assuntos abordados ao longo da presente dissertação. No **Capítulo 2** estão descritos os procedimentos empregados para a realização do trabalho, na forma de Materiais e Métodos. Os três artigos produzidos a partir dos resultados obtidos estão apresentados no **Capítulo 3**, e o **Capítulo 4** compreende uma discussão geral do trabalho realizado, assim como as conclusões obtidas a partir do mesmo.

CAPITULO 1

1 REVISÃO BIBLIOGRÁFICA

1.1 Planta de urucum

O urucuzeiro (*Bixa orellana* L.) é uma árvore de clima tropical, com uma altura de 3 - 5 m, tem folhas simples, pecioladas, membranáceas, glabras, de 8 – 11 cm de comprimento. As flores são brancas ou ligeiramente rosadas reunidas em panículas terminais, são emitidas com maior intensidade em duas épocas do ano, e assim definem a safra e a produtividade da planta (LOPES, 2003).

Os frutos do urucum são cápsulas arredondadas também chamados de cachopas que, podem chegar até 5 cm de largura. A coloração da sua superfície varia do verde ao vermelho intenso e se encontra revestida de espinhos flexíveis (Figura 1) (CARVALHO *et al.*, 2010).

Dentro dos frutos encontram-se as sementes cobertas por arilo vermelho, e são utilizadas tanto para a obtenção de mudas como para produção de corantes. Em média os frutos bem desenvolvidos possuem entre 40 – 50 sementes (Figura 1) (LORENZI, 2000).



Figura 1. Frutos e sementes de urucum (*Bixa orellana* L.). Fonte: (SANTOS *et al.*, 2014)

O pericarpo das sementes encontra-se revestido por uma resina vermelha, que é a principal substância responsável pela faixa de pigmentação do corante (vermelho,

alaranjado, amarelo). Pigmentos com diferentes características podem ser obtidos a partir das sementes, dependendo do método de extração utilizado (MANTOVANI *et al.*, 2013; SANTOS *et al.*, 2014).

1.2 Carotenoides nas sementes de urucum

1.2.1 Bixina

A bixina, é um tipo de carotenoide (6-metil hidrogênio 9'-*cis*-6,6'-diapocaroteno-6,6'-dioato) *cis*-bixina (Figura 2) que se define como apocarotenoide por ter um esqueleto com menos de 40 átomos de carbono, resultante da perda de moléculas de uma ou ambas as extremidades do carotenoide original. É responsável por mais de 80% do teor total de carotenoides em sementes de urucum, e só tem sido encontrada nestas sementes. A quantidade de pigmentos vermelhos em sementes de urucum varia 1,5 – 4,0 %, de acordo com variedade (MERCADANTE, 2007). O extenso sistema de ligações duplas conjugadas presentes na estrutura química da bixina é responsável da atividade antioxidante, mas também pela baixa estabilidade da molécula sobre condições de processamento e de armazenamento, tais como uma calor, luz e oxigênio (BARBOSA *et al.*, 2005).

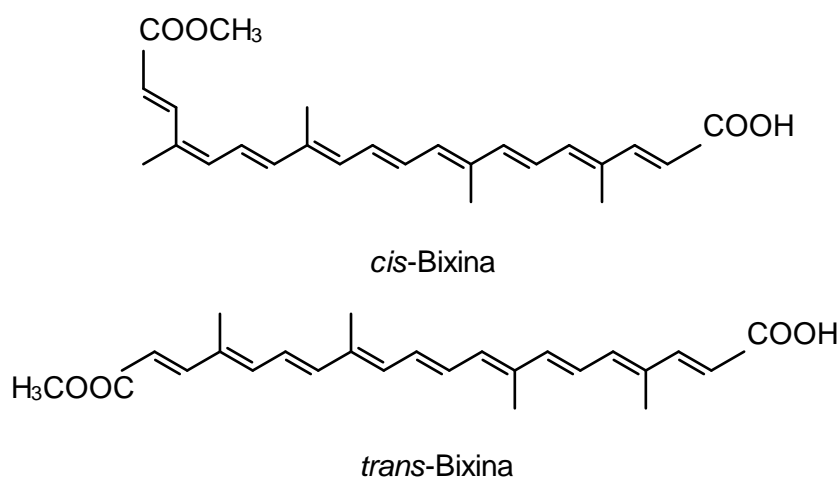


Figura 2. Estrutura da bixina nas formas *cis*- e *trans*. Fonte: (SMITH, 2006)

O mecanismo de biossíntese da bixina ainda segue em estudo, porém nos últimos anos os resultados relacionados a investigações de biologia molecular sugerem sua formação a partir de uma modificação oxidativa do licopeno, com a participação das enzimas aldeído oxidase e metiltransferase (MATTHEWS E WURTZEL, 2007).

1.2.2 Norbixina

A norbixina é um apocarotenoide (9'-*cis*-6,6'-diapocaroteno-6,6'-ácido dioico) *cis*-norbixina (Figura 2), um derivado desesterificado da bixina, encontrado em pequenas quantidades nas sementes de urucum, mas que se forma em condições alcalinas a partir da bixina mediante um processo de saponificação (HAGIWARA *et al.*, 2003), que consiste na desesterificação das moléculas de ambas extremidades do carotenoide mediante uma hidrólise básica causada pela adição de uma base forte.

Esse ácido dicarboxílico livre, através de uma dissociação química pode ser obtido com uma solução aquosa-alcalina como o hidróxido de sódio (NaOH) ou potássio (KOH), para formar o seu respectivo sal (GIULIANO *et al.*, 2003; SILVA *et al.*, 2009).

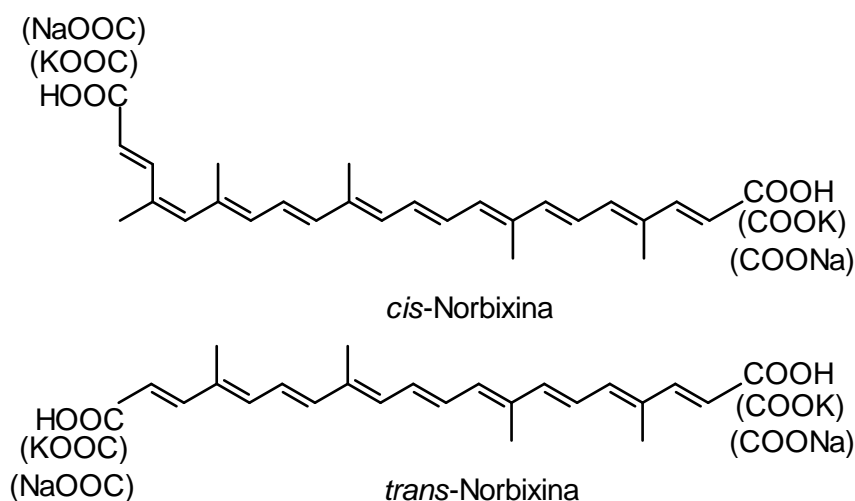


Figura 3. Estrutura da norbixina nas formas *cis*- e *trans*. Fonte: (SMITH, 2006)

1.2.3 Métodos de extração dos pigmentos de urucum

Existem alguns métodos estabelecidos para extrair os pigmentos a partir das sementes de urucum, sendo os mais utilizados: a extração alcalina (sal de norbixina), a extração com óleo (bixina) e a extração com solventes, tais como acetato de etila, etanol, clorofórmio e acetona, para se obter produtos com uma pureza mais elevada. Uma descrição de cada método de extração dos pigmentos está apresentada na Tabela 1. Estes corantes diferem na solubilidade e pigmentação (JECFA, 2006).

Uma intensa coloração vermelha nos extratos indica a presença de bixina concentrada, que é lipossolúvel, enquanto uma coloração amarela indica predominância de norbixina (SANTOS *et al.*, 2014).

Tabela 1. Métodos de extração de corantes de urucum.

Tipo de Extrato	Descrição	Pigmento
Extração com óleo	Imersão das sementes em óleo comestível a 70°C e submetidas ao atrito para remoção do pericarpo. Em seguida, a fração líquida é aquecida e filtrada; Mistura de finas partículas do pericarpo com óleo comestível;	Bixina
Suspensão em óleo	Extração com um solvente (acetona, por exemplo) seguida de lavagem com hexano; Extração com mono- e diglicerídios, ácidos graxos ou Propilenoglicol;	Bixina
Extração com álcali	A partir das sementes, onde o pericarpo é extraído na presença de álcali sob agitação e aquecimento; A partir da extração com solvente, seguida de hidrólise com álcali.	Norbixina

Adaptado de: (PRESTON E RICKARD, 1980).

O extrato de bixina apresenta relativamente um baixo custo de produção e não tem toxicidade quando aplicada em baixas concentrações (JNIOR *et al.*, 2012), além disso, tem características pouco comuns, tais como a possibilidade de obtenção de extratos solúveis em água e solúveis em óleo a partir da mesma fonte através de uma saponificação, e uma melhor afinidade em sua aplicação devido a sua propriedade de se ligar a certas proteínas presentes nos alimentos (ALVES *et al.*, 2006).

Alguns estudos têm sido realizados para avaliar os fatores que influenciam o método de saponificação da bixina, para assim estabelecer os valores de cada parâmetro e desta maneira otimizar a extração, com o fim de aumentar os rendimentos e a pureza do sal de norbixina (RIOS E MERCADANTE, 2004; NACHTIGALL *et al.*, 2009).

Em geral, a conversão da bixina em norbixina e seu sal, depende principalmente da concentração da base, ou seja, quanto mais concentrada a base mais completa será

a conversão. Um tempo de extração mais prolongado tem efeitos positivos na conversão da bixina em norbixina, porém podem também ocasionar degradação destes compostos. Quando temperaturas mais elevadas são utilizadas, há um aumento no rendimento de extração dos pigmentos, mas é necessário usar soluções alcalinas mais concentradas para não ter deficiências na reação de hidrólise da bixina em norbixina (SILVA *et al.*, 2009).

Contudo, para a obtenção de bixina e norbixina com alto teor de pureza, têm sido utilizados métodos de cristalização. Nos estudos realizados por (RIOS E MERCADANTE, 2004; LOBATO *et al.*, 2015), foram isolados padrões de bixina e norbixina. Foi utilizado o método de extração com solventes com indicação da eficiência do solvente acetato de etila em determinadas condições de volume de solvente, tempo e número de extrações necessárias para otimização da metodologia desenvolvida. A conversão de bixina em norbixina, durante a saponificação, foi de 100%. Como resultados foram obtidos cristais de bixina com alto teor de pureza (98 - 98,7%) e norbixina, purificada por coluna de sílica-gel, com pureza de 93%. O método utilizado mostrou ser simples e de baixo custo. Em ambos os estudos a verificação da pureza dos cristais da bixina e norbixina foi feita através de Cromatografia Líquida de Alta Eficiência (CLAE) conectada a um detector de arranjo de díodos.

1.3 Corante de urucum comercial

Na aplicação industrial, os extratos de urucum (bixina e norbixina) são usados como aditivo colorífico em alguns alimentos processados e podem melhorar as características sensoriais dos produtos. Os corantes de urucum apresentam uma vantagem econômica, pois são relativamente baratos em comparação com outros pigmentos naturais (CARDARELLI *et al.*, 2008).

Os corantes obtidos se classificam como lipossolúveis ou hidrossolúveis, segundo método de extração utilizado, e contém como pigmentos os compostos bixina e norbixina, respetivamente. A Tabela 2 apresenta os teores de pigmentos nos corantes de urucum de acordo com o método de preparação (SMITH, 2006).

Misturas de urucum com outros pigmentos naturais como, por exemplo, a curcumina, estão comercialmente disponíveis em extrato solúveis em água e em óleo. Seu uso é comum para dar um tom clássico de baunilha em sorvetes e também em margarinas ou queijos, onde o propósito é replicar o efeito colorífico do β -caroteno. As

misturas também podem ser usadas para criar tons da cor que são melhores dos que oferecem algumas marcas específicas de corantes sintéticos (KENDRIC, 2012).

Tabela 2. Teores de pigmento nos corantes de urucum segundo o método de extração.

Tipo de Corante	Preparação	Teor
Lipossolúvel	Soluções em óleo	0,05 – 1,0 % Bixina
	Suspensões em óleo	0,1 – 8,0 % Bixina
Hidrossolúvel	Soluções em água	0,1 – 4,0 % Norbixina
	Pó solúvel em água	1,0 – 15,0 % Norbixina

Adaptado de: (SMITH, 2006).

1.4 Estabilidade

Muitos aspectos da preparação de alimentos causam efeitos na estabilidade dos carotenoides. A condição de armazenamento, embalagem, congelamento, composição lipídica, componentes antioxidantes adicionais, matrizes de alimentos específicos, o contato com alimentos ácidos, área superficial, porosidade e oxidação enzimática durante o corte, descascamento, obtenção de polpa e suco podem contribuir para a variabilidade nos níveis finais de carotenoides nos alimentos (ARSCOTT, 2013).

1.4.1 Estabilidade térmica

O tratamento térmico e mecânico de frutos e vegetais tem o potencial de melhorar a biodisponibilidade de carotenoides devido ao rompimento da matriz das estruturas celulares; no entanto, também podem causar perda significativa desses compostos e provocar uma isomerização *cis – trans*. A temperatura elevada (calor) é o principal fator que afeta a integridade dos carotenoides durante o processamento de alimentos e pode produzir compostos voláteis e não-voláteis (ARSCOTT, 2013). A quantificação da norbixina por espectrofotometria está baseada na medida de absorvância, esse valor depende das ligações conjugadas presentes nas moléculas de norbixina e é afetado pela deslocalização dos π -elétrons ao longo da estrutura química deste polieno. Durante exposição às altas temperaturas, a hidrólise da norbixina em soluções aquosas pode ser responsável pela quebra dos enlaces duplos conjugados e,

portanto, da redução da absorvância (BITTENCOURT *et al.*, 2005; SCOTTER, 2009; DIAS *et al.*, 2011). No estudo realizado por GUAN E ZHONG (2014), foi encontrado que a goma arábica inibiu a hidrólise da estrutura da norbixina eficientemente durante o aquecimento. Adicionalmente, esse polímero impediu a precipitação da norbixina em condições ácidas (pH = 3,0 – 4,0).

No caso dos carotenoides, este tipo de tratamento conduz a uma redução da sua atividade ou a sua transformação em produtos de degradação com cores e propriedades diferentes. Um dos processos para a produção de extratos de urucum utiliza óleos comestíveis e aplicam-se temperaturas elevadas, que podem atingir 125 °C sob vácuo. Sob tais condições de operação, os principais compostos obtidos incluem o isômero de *trans*-bixina e um produto de degradação térmica de cor amarelo. A Figura 4 apresenta um esquema da degradação térmica da *cis*-bixina (RIOS *et al.*, 2005).

No estudo conduzido por RIOS *et al.* (2005) foi avaliada a estabilidade térmica da bixina em sistemas modelos aquosos (água/etanol (8:2)) usando temperaturas de 70 – 125 °C, como resultados da avaliação da degradação foram obtidos valores de constantes cinéticas de velocidade para a formação dos produtos primários da bixina.

Por outro lado, SCOTTER *et al.* (2001) avaliaram a cinética e rendimentos na formação de produtos coloridos e aromáticos resultantes da degradação térmica de urucum em alimentos. Como resultados relataram que a degradação térmica do principal composto, a *cis*-bixina, é termodinamicamente simples. A norbixina degrada mais lentamente, mas de forma semelhante e apenas baixos níveis de produtos de degradação foram observados durante o aquecimento.

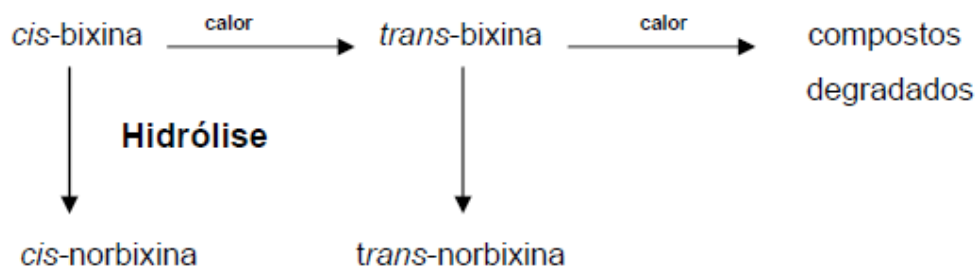


Figura 4. Esquema da degradação de *cis*-bixina. Fonte: (SILVA *et al.*, 2007)

A decomposição térmica do sal de norbixina obtido após a hidrólise da bixina foi analisado por análise termogravimétrica a diferentes taxas de aquecimento na faixa de

25 a 900 °C. As curvas de calorimetria de varredura diferencial (DSC) indicaram que as reações de decomposição térmicas ocorreram na fase sólida (<280 °C) (SILVA *et al.*, 2007).

No estudo realizado por LOBATO *et al.* (2015), foi avaliada a estabilidade térmica das nanocápsulas de bixina em um sistema aquoso água/etanol (8:2), onde as alíquotas desta solução foram aquecidas em temperaturas de 65, 80 e 95 °C. Os experimentos de aquecimento foram realizados com bixina livre e bixina nanoencapsulada. Como resultado foi observado que a perda da bixina livre ocorreu mais rapidamente do que a da bixina nanoencapsulada, de acordo com os maiores valores de constantes de velocidade e as energias de ativação apresentadas. As energias de ativação para as nanocápsulas foram superiores aos da bixina livre, o que sugere que existe um aumento da estabilidade quando é realizado o encapsulamento. Por tanto o encapsulamento permite melhorar a estabilidade da bixina quando exposta às altas temperaturas.

As mudanças que ocorrem após o processamento térmico de sistemas alimentares muitas vezes são monitoradas por diferentes parâmetros, como o conteúdo de carotenoides totais (portanto, isomerização e oxidação são subestimados), carotenoides individuais (mudanças globais podem ser dispensadas) e parâmetros de cor CIELAB (sem informações no mecanismo de degradação de carotenoides) (BORSARELLI E MERCADANTE, 2010).

1.4.2 *Ação antioxidante*

Além de sua relevância na fotossíntese, os carotenoides têm outras funções biológicas, das quais a interação com oxigênio ativo é fundamental. Os carotenoides presentes nos alimentos podem ter efeitos benéficos nos seres humanos, com participação importante na proteção de células atuando como antioxidante contra radicais livres e sequestro de oxigênio singlete (1O_2) devido ao seu longo sistema de duplas ligações conjugadas (RIOS, 2004). Enquanto o número das ligações duplas conjugadas em um carotenoide for maior, a eficiência de desativação de 1O_2 aumenta (CONN *et al.*, 1991).

Porém, esta estrutura característica de um carotenoide com a presença de várias ligações duplas conjugadas na cadeia e o cromóforo são os responsáveis pelas propriedades de absorção de luz, e também pela suscetibilidade à degradação frente a

fatores como altas temperaturas, pH baixo, a luz, presença de espécies reativas de oxigênio, entre outros (BORSARELLI E MERCADANTE, 2010).

Vários estudos têm sido realizados para avaliar a interação entre o carotenoides e oxigênio singlete, no entanto, em todos os casos, foi observado um fotobranqueamento dos carotenoides com a formação de vários produtos de oxidação, de acordo com o tempo de fotosintetização. A degradação química dos apocarotenoides (bixina, norbixina e seus isômeros) ocorre em soluções aquosas pela reação com oxigênio singlete, devido a sua conjugação conjugada (SPERANZA *et al.*, 1990; MONTENEGRO *et al.*, 2002).

A cinética de transformação fotossintética da bixina em uma mistura de solventes orgânicos foi avaliada por MONTENEGRO *et al.* (2004). Como resultado o estudo mostrou que nos processos de fotossensitização a bixina isomeriza para all-*trans*-bixina, independente da presença de oxigênio, com base em um mecanismo de transferência de energia, com formação da bixina oxidada, como o precursor de isomerização. Na presença de oxigênio singlete os produtos de isomerização são lentamente degradados, o que resulta em pequenas mudanças de cor na solução. Por conseguinte, sob as condições fotossensitizadas, a cor do sistema que contém bixina pode ser mantida, enquanto no caso do β -caroteno essa cor poderia desaparecer.

No estudo desenvolvido por RIOS *et al.* (2007), através de técnicas de calorimetria fotoacústica demonstraram que a bixina oxidada ou fotoquimicamente em estado triplete têm uma energia de ativação que pode interferir positivamente na sua capacidade para sequestrar 1O_2 .

Foi realizado por RIOS *et al.* (2009) um estudo para avaliar a modulação de espécies reativas de oxigênio (ROS) produzido pela interação DNA humano e cisplatina em um modelo experimental, através da aplicação dos carotenoides bixina e licopeno purificados e extraídos de fontes alimentares naturais. Os resultados demonstraram que a geração de ROS consequente da interação do DNA-cisplatina, foi inibida pelo licopeno e pela bixina de acordo com a concentração dos pigmentos. O licopeno e a bixina inibiram a produção de ânion superóxido e outras ROS. A formação de quantidades significativas de isômeros ou produtos de degradação de ambos os carotenoides não foi observada após a eliminação das ROS. Uma vez que estes estudos têm sugerido que os efeitos secundários resultantes do tratamento do câncer com cisplatina podem ser prevenidos ou reduzidos por carotenoides, a ingestão destas substâncias pode ser

útil para reduzir ou evitar os danos mediados pelas ROS. No entanto, os estudos ainda são necessários porque os mecanismos da ação antioxidante dos carotenoides não são totalmente conhecidos.

No estudo realizado por KIOKIAS E GORDON (2003) para avaliar a estabilidade da norbixina, verificou-se que este carotenoide pode retardar a deterioração oxidativa, tanto dos óleos e emulsões de óleo-água, e a ação foi particularmente mais eficaz nas emulsões do que outros compostos como a bixina, o β -caroteno, o palmitato de ascorbilo, o α -tocoferol e o ácido ascórbico. Misturas de norbixina com ácido ascórbico, palmitato de ascorbilo e δ - ou α -tocoferol aumentou o efeito antioxidante em relação ao apresentado pelos antioxidantes fenólicos nos óleos e emulsões, e efeitos sinérgicos foram observados com os tocoferóis e ácido ascórbico no sistema de emulsão.

A avaliação da estabilidade de nanocápsulas de bixina livre e de nanocápsulas em modelos de sistemas aquosos de água/etanol (8:2) durante a exposição à luz, na presença e ausência de oxigênio e sensibilizadores em temperaturas de 5 – 15 °C foi realizada por LOBATO *et al.* (2015). Como resultado foi obtido que as nanocápsulas de bixina exibiram maior energia de ativação do que a bixina livre em ambos os experimentos, o que sugere que o encapsulamento de bixina é responsável pelo aumento da estabilidade.

1.5 Encapsulamento

A tecnologia de encapsulação tem sido usada na indústria de alimentos para fornecer componentes que tenham uma barreira eficaz contra as interações ambientais e químicas, para aumento da estabilidade à temperatura, umidade, oxidação e exposição à luz, enquanto se prolonga a sua vida de prateleira (CORONEL-AGUILERA E SAN MARTÍN-GONZÁLEZ, 2015).

O encapsulamento é definido como um processo em que o material do núcleo (gota líquida, partícula sólida, ou bolhas de gás) é aprisionado (revestido ou embebido) em um material de grau alimentício, para obter como resultado um produto encapsulado com propriedades úteis. O material do núcleo que pode ser composto por apenas um ou vários ingredientes, representa o material revestido, ativo ou encapsulado. Por outro lado, o material externo da cápsula pode se apresentar em frações individuais ou misturas de vários componentes e é conhecido como agente de encapsulação, material de revestimento ou matriz. Os produtos alimentares que podem se beneficiar com o

encapsulamento incluem vitaminas, corantes e nutrientes (DE MARCO *et al.*, 2013; CHRANIOTI E TZIA, 2015).

A microencapsulação de pigmentos permite melhorar a sua estabilidade em condições adversas de armazenamento e assim aumentar a sua vida de prateleira, ajuda na solubilização e, portanto facilita a sua incorporação em alimentos (FAVARO-TRINDADE *et al.*, 2008). Existem várias técnicas que são eficazes na microencapsulação de ingredientes alimentares, destacando o leite fluidizado, a liofilização, a secagem por atomização (*spray drying*), a inclusão molecular e a inclusão em lipossomas (AZEREDO, 2005).

1.5.1 Agentes de encapsulação

A natureza do material de encapsulação é um fator muito importante em relação à estabilidade dos compostos encapsulados. É difícil encontrar um agente encapsulante que possa reunir todas as propriedades ideais, ter uma boa emulsificação, ter capacidade para formar películas, ser biodegradável, apresentar baixa viscosidade e baixa higroscopicidade, assim como um baixo custo. Portanto, geralmente na prática é usada comumente uma mistura de vários componentes (AZEREDO, 2005).

Os agentes de encapsulação podem ser selecionados entre uma ampla variedade de polímeros naturais ou sintéticos, fator que depende do material de núcleo ou ingrediente a ser revestido e das características desejadas nos produtos finais encapsulados. Os materiais utilizados principalmente na indústria de alimentos como agentes de encapsulação incluem polímeros de carboidratos, proteínas, lipídios e gomas, sendo os polímeros de carboidratos os mais amplamente utilizados (MURUGESAN E ORSAT, 2012).

As maltodextrinas são produtos de amido hidrolisado com diferentes pesos moleculares, e estas matrizes são comumente utilizadas como agentes de microencapsulação. Eles são classificados pelo seu equivalente em dextrose (DE) e devido às suas propriedades físico-químicas e de baixo custo são amplamente utilizados na indústria alimentar. Tanto materiais hidrofílicos como hidrofóbicos podem ser microencapsulados com maltodextrina (SOUSDALEFF *et al.*, 2013)

Uma classe de material muitas vezes explorado por suas capacidades de encapsulamento são os hidrocoloides, ou mais comumente conhecidos como, gomas.

Estes compostos são polímeros de cadeia longa que se dissolvem ou dispersam em água para se obter um espessamento ou efeito de aumento da viscosidade. As gomas são geralmente utilizadas como agentes de texturização, mas os seus efeitos secundários incluem capacidades de encapsulamento, estabilização de emulsões, controle de cristalização e a inibição da sinérese. Além disso, algumas das gomas também são capazes de formar géis (CHRANIOTI E TZIA, 2015).

A goma arábica tem sido o agente utilizado por muitos anos na secagem por atomização, uma vez que é atóxico, inodoro, insípido, com excelente capacidade de emulsificação, de baixa viscosidade em solução aquosa e excelentes propriedades de retenção volátil (GABAS *et al.*, 2007).

As maltodextrinas são amplamente utilizadas na encapsulação de compostos bioativos para protegê-los de alterações oxidativas e os efeitos da temperatura. Normalmente, é uma opção de baixo custo, mas têm baixa capacidade emulsificante e baixa viscosidade em concentrações muito elevadas e têm sido estudados como possíveis substitutos para goma arábica em emulsões atomizadas ou utilizado em conjunto com a goma arábica para satisfazer as propriedades requeridas para o material de encapsulação (RAMÍREZ *et al.*, 2015; AZEREDO, 2005).

1.5.2 *Processos de encapsulamento na indústria de alimentos*

A técnica de secagem por atomização é a técnica mais utilizada na indústria de alimentos, pois apresenta uma alta produtividade e ótima relação custo – benefício e os equipamentos são mais disponíveis, em comparação com a maioria das outras técnicas (DE MARCO *et al.*, 2013).

O processo de secagem envolve a atomização de emulsões em um meio de secagem a alta temperatura, o que causa uma evaporação de água muito rápida, e conseqüentemente este procedimento permite que um material seja aprisionado ou revestido por um agente encapsulante (FAVARO-TRINDADE *et al.*, 2008).

A técnica de secagem por congelamento ou liofilização é um dos processos mais úteis para a secagem de substâncias termo-sensíveis que são instáveis em soluções aquosas. Neste processo, o material congelado é submetido a uma pressão abaixo do ponto triplo (a 0 ° C, pressão: 610 Pa) e aquece-se para provocar a sublimação do gelo a vapor. Um processo de liofilização bem sucedido preserva a maior parte das

propriedades das matérias-primas iniciais, tais como forma, dimensões, aparência, sabor, cor, textura e atividade biológica (RAMÍREZ *et al.*, 2015).

No entanto, a liofilização é um processo lento e caro, sendo que o período necessário para a desidratação é geralmente de 20 a 24 h. Um longo tempo de processamento provoca demandas de energia adicional para operar as unidades de compressores e refrigeração, o que torna o processo muito caro para uso comercial (SAIKIA *et al.*, 2015).

1.6 Aplicações do urucum na indústria alimentícia

Algumas formulações comerciais dos extratos de urucum têm sido aprovadas para ser usadas como aditivos alimentares em diversas aplicações, tal como se amostra na Tabela 3. (SOCACIU, 2007). A bixina ocupa o segundo lugar entre os corantes naturais mais utilizados na indústria (BOUVIER *et al.*, 2003).

Tabela 3. Principais formas e aplicações dos corantes do urucum.

Pigmento	Componentes da formulação	Aplicação
Norbixina 2 a 4%	Extrato de urucum, Água, Hidróxido de Potássio;	Queijos, Sorvetes, Produtos panificados;
Norbixina 2,5 a 20%	Extrato de urucum, Carbonato de Sódio;	
Norbixina 2,5%	Extrato de urucum, Polisorbato 80, Propilenoglicol, Hidróxido de Potássio, mono e diglicerídeos;	Sobremesas, Iogurtes, Bebidas;
Bixina 1%	Extrato de urucum em óleo vegetal, mono e diglicerídeos, Propilenoglicol, Hidróxido de Potássio;	Manteigas, Óleos, Margarinas, Queijos maturados, Produtos ricos em gordura;
Bixina 2%	Extrato de urucum em óleo vegetal.	

Adaptado de: (SOCACIU, 2007).

Em relação ao consumo do corante, cada carotenoide tem uma dose recomendada pela Joint FAO/WHO Expert Committee on Food Additives (JECFA). A ingestão diária aceitável (IDA) para o extrato de urucum é de 250 mg/Kg de peso corporal, para a bixina a IDA é de 0 – 12 mg/Kg de peso corporal, e para o caso do sal de norbixina a IDA é de 0 – 0,06 mg/Kg de peso corporal (JECFA, 2006).

Para as indústrias alimentares e de medicamentos os extratos de urucum poderiam ser uma interessante alternativa como corantes naturais, devido à sua potencial utilização como um substituto para a tartrazina, que é um corante sintético proibido em muitos países (TAHAM *et al.*, 2015).

Embora os corantes naturais de urucum ainda não têm sido usados como aditivos alimentares em bebidas, a norbixina seria uma alternativa por ser um composto hidrossolúvel. As bebidas isotônicas comercializadas no mercado atual têm como ingredientes os corantes sintéticos ou artificiais tais como Vermelho 40, Tartrazina ou Azul Brilhante. Estas substâncias podem apresentar problemas de alergia nos consumidores, então uma possível aplicação de corantes naturais obtidos de urucum nestas bebidas poderia acarretar em efeitos benéficos para a saúde humana.

CAPITULO 2

2 MATERIAL E MÉTODOS

O presente estudo foi desenvolvido no Laboratório de Compostos Bioativos do Instituto de Ciência e Tecnologia de Alimentos (ICTA) da Universidade Federal do Rio Grande do Sul.

2.1 Isolamento do padrão de bixina

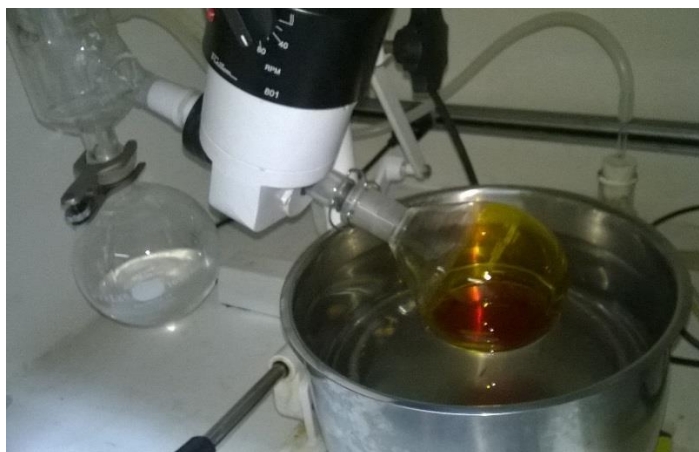
As sementes de urucum foram adquiridas no comércio local de Porto Alegre - RS e armazenadas sob temperatura ambiente até o momento dos experimentos. Os polissacarídeos comestíveis Goma Arábica SYNTH (Porto Alegre, RS, Brasil) e Maltodextrina DE20 Vallens (Porto Alegre, RS, Brasil) foram usados como agentes encapsulantes.

A extração de bixina foi realizada de acordo com RIOS E MERCADANTE (2004) para a obtenção de um extrato com menor teor de impurezas possível. O método se baseia no princípio de lavagens prévias das sementes de urucum com hexano e metanol para eliminação de lipídios e compostos polares, respectivamente. Para tal, foram utilizadas 25 g de sementes de urucum que foram lavadas duas vezes com 50 mL de hexano por 15 minutos, sendo o solvente descartado. Uma nova lavagem foi realizada com 50 mL de metanol durante 15 minutos por duas vezes, sendo as sementes filtradas e o solvente também descartado. Foram realizadas duas extrações dos pigmentos com 50 mL de acetato de etila cada por 15 minutos e o extrato obtido foi seco em evaporador rotatório (Fisatom M802, Brasil) ($T < 30^{\circ}\text{C}$) (Figura 5).

Na cristalização, o extrato seco com os pigmentos foi diluído em diclorometano e adicionado lentamente etanol absoluto na proporção em volume de 1:4. Em seguida, a mistura permaneceu em banho de gelo por 5 minutos e posteriormente foi armazenada a -18°C por 12 horas para a formação dos cristais de bixina. A pureza dos cristais foi verificada por Cromatografia Líquida de Alta Eficiência (CLAE) e através do espectro no UV-visível da bixina de acordo com as áreas identificadas no comprimento de onda de 453 nm (Figura 5).



Lavagem – Extração



Secagem em evaporador rotativo



Formação de cristais de bixina

Figura 5. Imagens do processo de obtenção dos cristais de bixina

2.2 Isolamento do padrão de norbixina

O padrão de norbixina foi obtido de acordo com o procedimento descrito por RIOS E MERCADANTE (2004). No extrato isolado de cristais de bixina foi adicionado 50 mL de uma solução metanólica de KOH (10%) e o extrato foi armazenado em ausência de luz a 25°C por 12 horas para a saponificação da bixina, originando sais de norbixina. Após, os sais foram separados em um funil de separação através da adição de 50 mL de água destilada, 50 mL de éter etílico, extrato obtido da saponificação e 50 mL de acetato de etila. Em seguida, foi adicionado ácido acético glacial até a fase inferior alcançar pH 3,5 e haver transferência da norbixina (coloração vermelho intenso) para a fase superior. Posteriormente, foram feitas lavagens com água destilada até a fase inferior atingir pH 6,0. A fase superior contendo norbixina foi separada e concentrada

em evaporador rotatório (Fisatom M802, Brasil) ($T < 30\text{ }^{\circ}\text{C}$), originando um pó que foi armazenado a $-18\text{ }^{\circ}\text{C}$ (Figura 6).



Figura 6. Imagens do processo de obtenção dos cristais de norbixina

2.3 Análise de espectrofotometria UV/Visível

Primeiramente, a norbixina foi dissolvida em etanol absoluto (99,7%), e uma alíquota do extrato foi diluída em uma solução aquosa KOH (0,5%) que foi usada como padrão. Após isso, a norbixina foi quantificada com um espectrofotômetro (Shimadzu UV-1800, Japão) a 453 nm (ALVES et al., 2006). Foi desenvolvida uma curva padrão para a quantificação da norbixina na faixa de 2,30 a 27,64 $\mu\text{g/mL}$ com um fator de correlação (R^2) de 0,99. Para os cálculos foi usado o coeficiente de absorvidade ($E_{1\text{cm}}^{1\%} = 3473$) da solução padrão (YABIKU & TAKAHASHI, 1992) usando a seguinte equação:

$$X(\mu\text{g}) = \text{Abs} \times \gamma \times 106 / E \times 100 \quad (1)$$

Abs = Absorbância

γ = diluição (mL KOH 0,5%)

E = Coeficiente de absorvidade

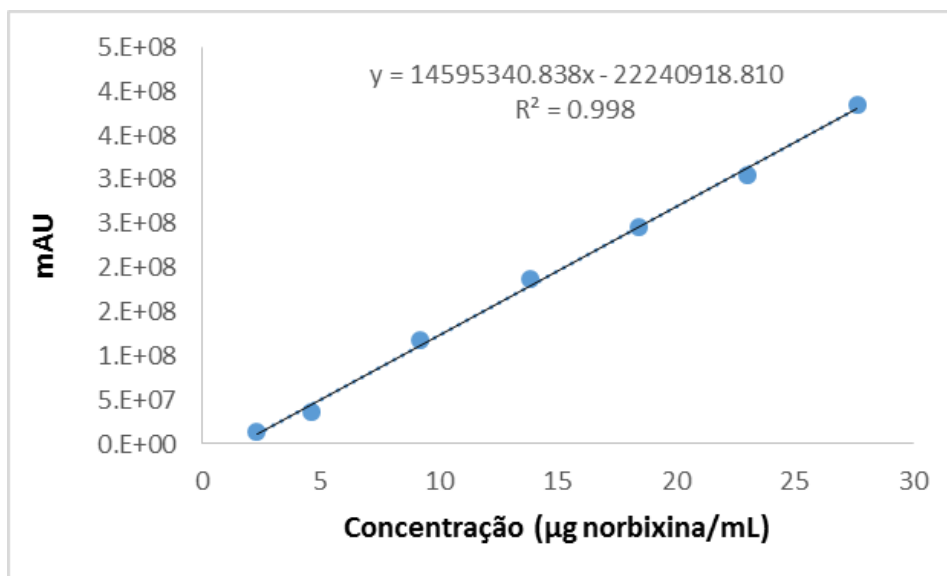


Figura 7. Curva padrão de norbixina

2.4 Microencapsulação dos cristais de norbixina

Para a preparação do núcleo, foi realizada uma dissolução de 20 mg de cristais de norbixina em 20 mL de etanol absoluto (99,7%). Por outro lado, uma quantidade de polissacarídeos foi dissolvida em 80 mL de água destilada mantidas sob agitação com agitador magnético e a 50°C para conseguir uma completa dissolução dos carboidratos. A maltodextrina e goma arábica foram adicionadas nas diferentes proporções MD:GA (100:0, 85:15, 65:35, 50:50, 35:65, 15:85, 0:100) até atingir 20% de sólidos solúveis totais. Após, foi adicionado o extrato de norbixina dissolvida em etanol na solução de polissacarídeos, cada formulação foi homogeneizada com agitador magnético por 30 min, finalmente foram armazenadas a 4°C (Figura 8).

A dispersão foi submetida a secagem por atomização em secador (Mini Spray Dryer Büchi B290, Suíça) com temperaturas de secagem de ar de entrada 150°C e de saída 75°C, respectivamente. O diâmetro do bocal de atomização foi 0,7 mm, a taxa de fluxo de ar de secagem 600 N L h⁻¹ e taxa de fluxo de alimentação 0,39 L h⁻¹ (Figura 8). Estas condições de operação foram usadas para todas as formulações e cada experimento foi realizado em duplicata. As MCN obtidas foram armazenadas imediatamente em frascos âmbar e mantidas sob refrigeração a 4°C.



Dissolução dos carboidratos



Formulações



Microencapsulação em spray dryer

Figura 8. Processo de microencapsulação dos cristais de norbixina

2.4.1 *Rendimento do processo*

O rendimento da microencapsulamento (RM) foi avaliado por gravimetria, usando a seguinte equação (FANG E BHANDARI, 2010):

$$RM(\%) = (\text{Peso das MCN (g)}/\text{Peso inicial de sólidos (g)}) \times 100 \quad (2)$$

RM = Rendimento de microencapsulamento

MCN = Microcápsulas de norbixina

2.4.2 Teor de umidade

Imediatamente após cada experimento de secagem por atomização a umidade das MCN foi analisada usando uma balança térmica MB25 (Ohaus, Brazil) (Figura 9). Uma amostra de 1 g foi colocada no prato e a água foi evaporada por aquecimento da amostra com luz halogena. Após 60 segundos, a umidade foi obtida e expressada como porcentual.



Figura 9. Avaliação da umidade das microcápsulas de norbixina

2.4.3 Eficiência de Microencapsulação (EM)

A EM é definida como a diferença entre a retenção de norbixina (RB) nas microcápsulas e a norbixina presente na área superficial das microcápsulas (RS). A avaliação da EM foi realizada seguindo o procedimento estabelecido por STOLL *et al.* (2016) com algumas modificações. Para realizar uma extração exaustiva da RS foram dissolvidas 100 mg de MCN em 1 mL de metanol/etanol em razão 1:1. A amostra foi homogeneizada por 2 min à temperatura ambiente em um vortex, após foi centrifugada (3000 x g, 10 min, 4°C) e o sobrenadante foi coletado e armazenado em frascos âmbar. Após, para quantificar a RB foi adicionado 1 mL de etanol/água destilada em razão 1:1 e mantidas sob agitação com um homogeneizador Ultra-turrax T25 (IKA, China) por 2 min à temperatura ambiente após foi feita uma centrifugação (3000xg, 10 min, 4°C) e o sobrenadante foi coletado e armazenado em frascos âmbar (Figura 10). Finalmente, a quantidade de norbixina presente em cada extrato de RS e RB foi avaliada por CLAE. Todas as análises foram realizadas em triplicata. A porcentagem de EM foi calculada de acordo com a equação:

$$EM\% = ((RB - RS)/RS) \times 100 \quad (3)$$

EM = Eficiência de microencapsulamento

RB = Retenção da norbixina no interior das microcápsulas

RS = Retenção da norbixina na superfície das microcápsulas

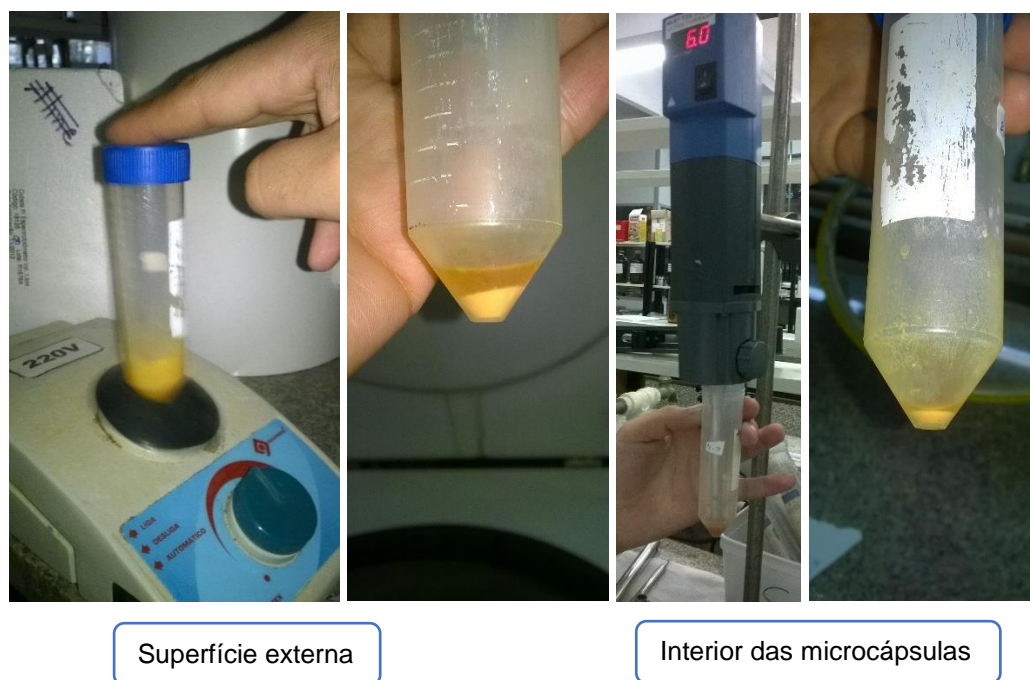


Figura 10. Extração da norbixina para avaliar eficiência de microencapsulamento

2.4.4 Atividade antioxidante método ABTS

A capacidade sequestrante ABTS das MCN foi determinada depois do processo de secagem por atomização, a extração da norbixina foi realizada de acordo com o procedimento descrito por STOLL *et al.* (2016) com modificações. Para isso 2,5 g de MCN (566 µg norbixina) foram diluídas em 2 mL de água destilada e misturadas em um vortex durante 2 min, após isso foram centrifugadas (3000xg, 10 min, 4°C). O sobrenadante foi coletado e colocado em frascos âmbar. Para os cristais de norbixina foi seguido o mesmo procedimento.

Posteriormente, a 2,2'-azino-bis (ácido 3-ethylbenzothiazoline-6-sulfônico) (ABTS) capacidade sequestrante das amostras de MCN foi determinada de acordo com o método modificado, previamente descrito por RE *et al.* (1999). O radical ABTS⁺ foi preparado através da reação de 5 mL ABTS (7mM) com 88 µL de persulfato de potássio (140 mM), ambas soluções foram previamente preparadas. A mistura foi armazenada a

temperatura ambiente em ausência de luz por 16 horas antes de ser usada. A solução de ABTS⁺ foi diluída em etanol até atingir uma absorvância de $0,700 \pm 0,020$ a 734 nm avaliada mediante o uso de um espectrofotômetro CPS-240A (Shimadzu, Japão). Após, 100 µL do extrato de norbixina foi adicionado em 1 mL do ABTS⁺ diluído e valor de absorvância foi registrado após 6 min.

Cada análise foi realizada em triplicata. A curva padrão foi desenvolvida com diferentes concentrações de Trolox (11 a 176 µM) e os resultados foram expressados como µmol TE/g amostra seca.

2.4.5 Solubilidade em água

A solubilidade em água foi definida como o nível no qual as MCN foram solubilizadas em médio aquoso e foi quantificada de acordo ao procedimento modificado por STOLL *et al.* (2016). Para isso, foram utilizadas placas de Petri previamente pesadas e colocadas em dessecador. Primeiramente, 2,5 g de MCN foram adicionadas em 30 mL de água destilada e a mistura foi homogeneizada por 2 min em um homogeneizador Ultra-turrax T25 (IKA, China). Após, a mistura foi colocada em banho maria a 25°C por 30 min e centrifugada (3500 x g, 15 min, 4°C). O sobrenadante foi coletado e colocado em uma placa de Petri. As placas foram levadas para estufa por 12 horas a 105°C. A quantidade de sólidos no sobrenadante foi expressada em porcentual para indicar o índice de solubilidade em água.

2.4.6 Análises de colorimetria

A cor das microcápsulas foi analisada no dia da aquisição (dia zero) e depois foram embaladas a vácuo e armazenadas a 4°C no abrigo da luz. A cor foi medida através de um colorímetro Chromameter CR-400 (Konica Minolta, Japão) segundo os parâmetros (L^* , a^* , b^*) de acordo com sistema CIELab (Figura 11). Foram determinados os parâmetros L^* que significa o nível de luminosidade (0 = branco; 100 = preto), a^* que estabelece a variação entre verde ($<a^*$) e vermelho ($>a^*$), e b^* , a variação entre azul ($<b^*$) e amarelo ($>b^*$). Um prato branco de cerâmica foi usado para calibrar o equipamento antes de realizar cada avaliação. O valor do ângulo Hue e fator Chroma, os quais indicam a tonalidade da amostra (0 ou 360° = vermelho, 90° = amarelo, 180° = verde, e 270° = azul) e a intensidade da saturação da cor, respectivamente, foram calculadas usando as seguintes equações (KIM *et al.*, 2002):

$$\text{Hue} = \tan^{-1} (b^*/a^*) \quad (4)$$

$$\text{Chroma} = (a^*)^2 + (b^*)^2 \quad (5)$$



Figura 11. Avaliação da cor das microcápsulas de norbixina

2.4.7 Distribuição de tamanho de partícula

Para avaliar a distribuição de tamanho de partícula (*Span*) foi usado o Mastersizer 2000 (Malvern Instruments Ltd., UK) acoplado com um módulo de dispersão em seco Sirocco 2000. O equipamento utilizou um procedimento baseado na técnica de difração de raios laser. O diâmetro volumétrico médio expressado como diâmetro D[4,3] se refere a um diâmetro médio ponderado do volume, assumindo partículas esféricas com o mesmo volume que as partículas reais. O *Span* foi calculado segundo a equação:

$$\text{Span} = (d(0.9) - d(0.1))/d(0.5) \quad (6)$$

Sendo $d_{0.9}$, $d_{0.1}$, e $d_{0.5}$ os diâmetros a 90, 10 e 50% da curva de distribuição de tamanho cumulativo, respectivamente.

2.4.8 Microscopia eletrônica de varredura (MEV)

A morfologia das micropartículas foi avaliada por MEV usando um microscópio eletrônico de varredura Zeiss EVO MA 10 (Carl Zeiss, Alemanha). Primeiramente, quantidades pequenas das MCN foram fixadas em stubs de alumínio com fita adesiva dupla-face e o conjunto foi recoberto com uma fina camada de ouro. As amostras foram escaneadas com voltagem de aceleração de 5 kV e ampliações de 2,50 kx, 10 kx e 20 kx. As imagens foram capturadas e gravadas.

2.4.9 Cromatografia Líquida de Alta Eficiência (CLAE)

Para verificação da pureza dos cristais de bixina e da norbixina, e quantificação de extratos isolados e obtidos das microcápsulas, foi usado o método desenvolvido por MONTENEGRO *et al.* (2004) utilizando um cromatógrafo líquido de alta eficiência HPLC 1100 (Agilent, USA), constituído de sistema quaternário de bombeamento de solventes, injetor automático, forno com controle de temperatura, degaseificador “on line”, e o detector de UV-visível. A separação dos pigmentos foi realizada em coluna C18 Spherisorb ODS-2 (150 x 4,6 mm), com 3 µm de diâmetro. A fase móvel utilizada foi composta de acetonitrila/ácido acético 2% (65:35), com fluxo de 1 mL/min e temperatura da coluna mantida a 29°C. O volume de injeção foi 5 µL. Os espectros foram adquiridos entre os comprimentos de onda de 250 e 600 nm e os cromatogramas processados no comprimento de onda de 472 e 450 nm para bixina e norbixina, respectivamente. O programa CHEMSTATION® foi usado para a aquisição e processamento de dados. Para cada injeção, todas as amostras foram pré-filtradas usando uma membrana PTFE modificada para solventes aquosos e orgânicos com um diâmetro de poro de 0,45 µm (Millipore, Brasil), cada injeção foi realizada em triplicata.

Para a quantificação da norbixina foi usada uma curva padrão analítica que foi desenvolvida previamente com diferentes concentrações de norbixina. Os valores dos limites de detecção e quantificação foram 0,0304 µg/mg e 0,0305 µg/mg, respectivamente e foram calculados de acordo com o procedimento descrito por ERTAŞ *et al.* (2007).

2.5 Avaliação da estabilidade térmica das microcápsulas

Os experimentos de aquecimento foram realizados em sistema modelo aquoso no escuro. Para isso foram colocadas 100 mg de MCN em tubos de centrifuga de 15 mL com tampa de rosca e espessura de parede de 1 mm, os tubos foram levados para banho de aquecimento a 60, 90 e 98°C. Periodicamente foi tomada uma amostra do banho e depositada imediatamente em banho de gelo. Após do esfriamento foi adicionado em cada tubo 1 mL de etanol/água (1:1, v/v) para extrair a norbixina das MCN, a mistura foi agitada por 2 min usando um ultra-turrax digital T25 (IKA, Brasil) e foi centrifugada (3000xg, 10 min, 4°C), o sobrenadante foi coletado. Para os experimentos com norbixina livre (não-encapsulada), os cristais purificados de norbixina foram dissolvidos em uma solução aquosa KOH 0,5% até atingir a concentração de 4.50 µg/mL; depois uma alíquota de 1 mL foi colocada em um frasco âmbar de 10 mL e

submetido ao mesmo procedimento de aquecimento das MCN. O teor de norbixina nas amostras de MCN e norbixina livre foi avaliado periodicamente por CLAE nos seguintes períodos: 0, 15, 30, 45, 60, 90, 120, 150, 180, 240 e 300 min durante os experimentos (Figura 12). Com os dados da concentração de norbixina foram determinados os parâmetros cinéticos usando o programa Origin 8.0 Pro (Origin Lab Co., USA). Adicionalmente foi calculado o tempo de vida média ($t_{1/2}$) de acordo com a seguinte equação (BUSTOS-GARZA *et al.*, 2013):

$$t_{1/2} = \frac{\ln 2}{k_t} \quad (7)$$

$t_{1/2}$ = tempo de vida media

k_t = Constante cinética

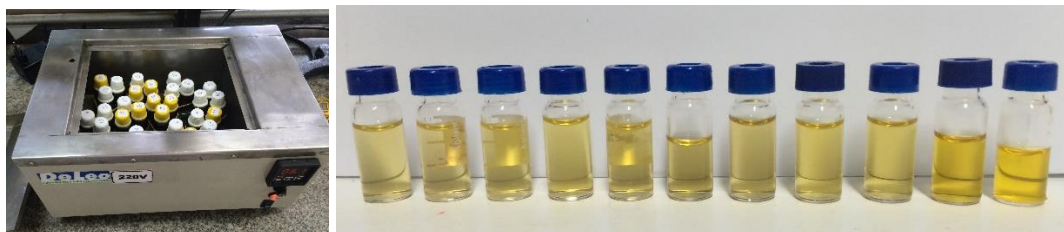


Figura 12. Avaliação da estabilidade térmica das microcápsulas de norbixina

2.6 Aplicação das microcápsulas obtidas: corante natural em bebidas isotônicas de tangerina

Após a avaliação da eficiência de encapsulamento das MCN e a caracterização; foram selecionadas as microcápsulas que apresentaram os melhores resultados (formulação com 100% de goma arábica como agente de encapsulação) para realizar a aplicação em uma matriz alimentícia e avaliar sua estabilidade sob condições aceleradas de armazenamento. Assim, bebidas isotônicas de tangerina foram produzidas e adicionadas de MCN como corante natural com o intuito de obter a cor desejada (laranja) neste tipo de bebida para propiciar a substituição do uso dos corantes sintéticos. Os cristais purificados de norbixina (não encapsulados) foram utilizados para fins de comparação.

2.6.1 Preparação de uma bebida isotônica de tangerina (BIT)

Previamente foi avaliada a cor de uma bebida isotônica comercial de tangerina para definir a quantidade de norbixina que devia ser adicionada BIT para obter um produto com valores similares dos parâmetros CIELab ($L^* = 48,27 \pm 0,01$; $a^* = 6,75 \pm 0,04$; $b^* = 22,76 \pm 0,01$), sem exceder o limite estabelecido para norbixina E160b ($0,005 \text{ g}/100 \text{ mL}$) como aditivo em bebidas não alcoólicas na legislação brasileira de alimentos (ANVISA, 2006). Após vários experimentos prévios, a concentração de norbixina que atingiu a tonalidade desejada (laranja) de acordo com a carta de cor CIELab ficou na faixa de $2,50$ a $2,90 \text{ } \mu\text{g}/\text{mL}$. Portanto, no estudo foi desenvolvida uma bebida dentro dessa faixa de concentração de norbixina. Para isso, foram preparadas duas soluções de bebida isotônica, cada uma em 1250 mL de água destilada com 50 g de açúcar, 875 mg de KCl, 1125 mg de NaCl, 375 mg de ácido cítrico, 375 mg de ácido ascórbico e $375 \text{ } \mu\text{L}$ de saborizante artificial de tangerina. Em uma solução de bebida isotônica foram dissolvidas 10 g de MCN até atingir uma bebida com uma concentração final de $2,86 \pm 0,02 \text{ } \mu\text{g}$ de norbixina/mL com o objetivo de obter uma BIT colorida com MCN (Isotônica-MCN). Posteriormente, como controle na outra solução de bebida isotônica foram adicionadas $650 \text{ } \mu\text{g}$ de cristais de norbixina até atingir uma bebida com uma concentração final de $2,56 \pm 0,05 \text{ } \mu\text{g}$ de norbixina/mL obtendo uma BIT colorida com cristais de norbixina (Isotônica-NC). As bebidas foram armazenadas a -4°C em ausência da luz (Figura 13).

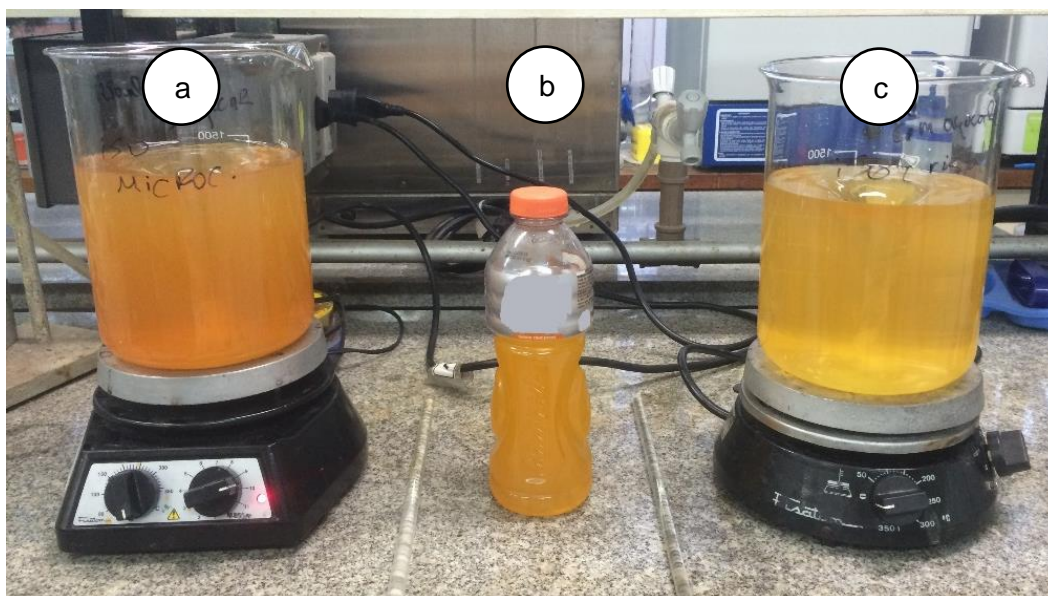


Figura 13. Bebidas isotônicas de tangerina: a) adicionadas de microcápsulas de norbixina; b) bebida comercial; c) adicionadas de cristais de norbixina

2.6.2 Acidez, pH e sólidos solúveis totais (°Brix)

Após a preparação as bebidas Isotônica-MCN e Isotônica-NC foram caracterizadas para obter o valor inicial de acidez, pH e °Brix. Durante o tempo de armazenamento periodicamente foi tomada uma amostra de cada bebida para caracterização. Para determinar a acidez, as amostras foram diluídas em água destilada, posteriormente foram adicionadas gotas de fenolftaleína (1%), e a mistura foi titulada com uma solução aquosa de NaOH 0,01 N até a virar a cor. O resultado foi expresso em mg ácido cítrico/L de amostra. A quantidade de sólidos solúveis totais foi avaliada usando um refratômetro portátil Pocket ATAGO model PAL-3 (Japão) e foi expresso em °Brix. Para avaliar o pH foi usado um pHmetro QUIMIS (Brasil).

2.6.3 Cor

A avaliação dos câmbios na cor das bebidas durante o armazenamento foi monitorada usando um colorímetro Chromameter CR-400 (Konica Minolta, Japão). As medidas foram registradas de acordo com os parâmetros L^* , a^* , b^* do sistema CIELab. Estes valores foram usados para calcular os fatores Chroma e Hue como mencionado no item 3.4.6 e adicionalmente foi calculado o valor da diferença de cor (ΔE) seguindo a equação (KIM *et al.*, 2002):

$$(\Delta E) = [(L_o^* - L^*)^2 + (a_o^* - a^*)^2 + (b_o^* - b^*)^2]^{1/2} \quad (8)$$

2.6.4 Estabilidade das bebidas sob condições aceleradas de armazenamento

Previamente foram realizados vários testes para avaliar a perda de cor nas bebidas durante armazenamento sob condições aceleradas, com o fim de estabelecer a temperatura e períodos de tempo dos ensaios experimentais. As MCN precisaram uma maior temperatura e tempo para começar a degradar do que quando a norbixina foi testada em sua forma não encapsulada (cristais). Portanto, foi estabelecida uma maior temperatura e tempo de armazenamento para os experimentos com amostras da bebida Isotônica-MCN; para os experimentos com sistemas não encapsulados foram usadas amostras da bebida Isotônica-NC. As bebidas foram distribuídas em garrafas plásticas transparentes com tampa rosca e volume de 30 mL em cada uma, sendo imediatamente adicionado nitrogênio para eliminar a presença de oxigênio. As garrafas foram fechadas e seladas com filme plástico de parafina. Para avaliar a influência do material de parede das MCN sob a estabilidade da norbixina dois conjuntos de

experimentos foram conduzidos em uma estufa de laboratório; cada um com uma temperatura específica. Para Isotônica-NC as amostras foram armazenadas em exposição à luz fluorescente (ca. 750 lux) ou em ausência da luz a $35 \pm 2^\circ\text{C}$ (Figura 14) e a concentração de norbixina (Figura 14), cor, °Brix, acidez e pH nas amostras foram avaliadas a 0, 30, 60, 180, 240, 300, 420 e 500 min durante o armazenamento. Para Isotônica-MCN as amostras foram armazenadas em exposição à luz fluorescente (ca. 750 lux) ou em ausência da luz a $40 \pm 2^\circ\text{C}$ e os mesmos parâmetros nas amostras foram avaliados a 0, 1, 3, 5, 7, 10, 12, 15 dias durante o armazenamento. Todos os experimentos foram realizados em triplicata.



Figura 14. Armazenamento de amostras de bebidas isotônicas de sabor tangerina adicionadas de microcápsulas e cristais de norbixina sob condições aceleradas (luz e calor)

A cinética de degradação da norbixina foi analisada usando a reação de primer-ordem como já foi reportado em outros estudos da estabilidade térmica da norbixina (SILVA *et al.*, 2007; SCOTTER, 2009). Para calcular os parâmetros cinéticos, a retenção de norbixina (%) nas bebidas foi plotada em relação ao tempo de armazenamento. A constante cinética de degradação (k_d) foi determinada a partir da primeira derivada destas curvas usando o programa Origin 8.0 (Origin Lab Co., USA) (DE ROSSO E MERCADANTE, 2007). Para o cálculo da retenção de norbixina e o tempo de vida média (BUSTOS-GARZA *et al.*, 2013) foram usadas as seguintes equações:

$$[\text{norbixina}] = [\text{norbixina}]_0 \times \exp(-k_t \times t) \quad (9)$$

$$t_{1/2} = \ln 2 / k_t \quad (10)$$

$t_{1/2}$ = Tempo de vida media

k_t = Constante cinética

t = tempo

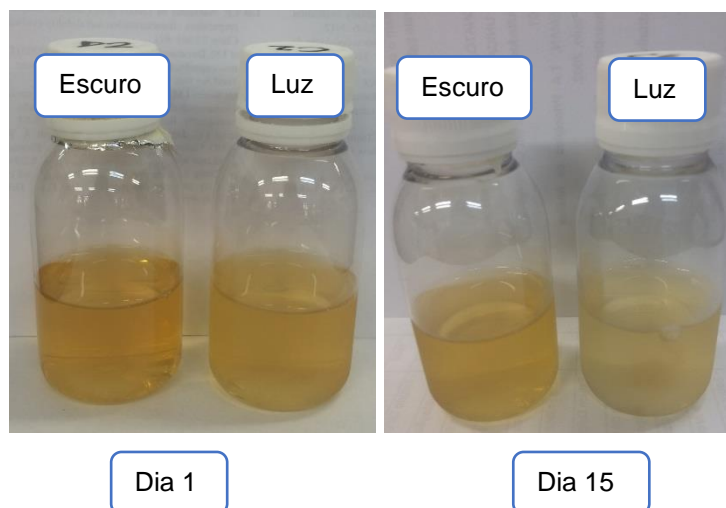


Figura 15. Amostras de bebida isotônica de tangerina adicionada de microcápsulas de norbixina ao início e fim do experimento

2.7 Análise Estatística

Para todos os cálculos de parâmetros estatísticos foi usado o programa STATGRAPHICS Centurion. A análise estatística (ANOVA) e o teste de comparação de médias de Tukey foram realizados para correlacionar os dados e estabelecer diferença estatisticamente significativa ($p < 0,05$) com um nível de 5% de significância. Todos os resultados foram registrados e expressados como média \pm desvio padrão.

CAPITULO 3

ARTIGOS

ARTIGO 1

**MICROENCAPSULATION EFFICIENCY OF NORBIXIN ENCAPSULATED BY
SPRAY-DRYING USING GUM ARABIC AND MALTODEXTRIN AS WALL
MATERIALS**

Artigo formatado de acordo com as normas da revista "Journal of Food Science"

Microencapsulation efficiency of norbixin encapsulated by spray-drying using gum arabic and maltodextrin as wall materials

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ABSTRACT

Norbixin is a carotenoid with antioxidant properties commonly used as a natural coloring in processed products; however, its chemical structure makes it susceptible to environmental factors such as light, oxygen, and temperature. Microencapsulation is used to improve the stability and solubility of the compound. The process efficiency depends on the encapsulation agents and operational parameters. In this study, gum arabic (GA) and maltodextrin (MD) were used in different proportions as wall materials. All formulations were prepared with the same core:wall material ratio. The formulation 0:100 (MD:GA) showed the highest microencapsulation efficiency with 40% of total soluble solids (TSS) (74.91% - 226.40 µg/g) and a good process yield (61.07%). The particle size distribution and scanning electron microscopy (SEM) of norbixin microcapsules (MCN) were analyzed, and a uniform particle diameter with homogeneity was obtained. MCNs showed high solubility (>90%), low moisture ($2.86 \pm 0.81\%$) content and high antioxidant activity (77.77 ± 0.59 µmol TE/g dry sample).

Keywords: Microencapsulation; annatto; norbixin; natural dyes; bioactive compounds; carbohydrate polymers.

1 Introduction

Norbixin, an apocarotenoid (9'-*cis*-6,6'-diapocarotene-6,6'-dioic acid) *cis*-norbixin, is found in small amounts in annatto seeds (*Bixa orellana* L.), where 80% of the pigments are bixin (Barbosa et al., 2005). However, this carotenoid can also be obtained through saponification of bixin by the use of alkaline solutions (NaOH or KOH) to form its respective salt in polar solvents (Scotter, 2009). Several methods have been developed and validated to obtain highly pure norbixin extracts from annatto seeds (Rios & Mercadante, 2004).

Bixin and norbixin are chemically similar compounds, however, exhibit differences in their solubility and stability due to its chemical structure (Scotter, 2009). Pigments with a high bixin or norbixin content are fat-soluble and water-soluble,

respectively. Commercially, this property defines its application in the food industry (Prabhakara Rao et al., 2005).

Annatto extracts are used as a natural coloring in butter, cheese, bakery products, oils, ice cream, sausages, extruded products, salad dressings, cereals, sodas and liquors, and they have a lower cost about other natural pigments (Cardarelli et al., 2008).

Besides its color capacity, norbixin has antioxidant properties due to its long system of conjugated double bonds (Rios & Mercadante, 2004). In contrast, this chemical structure is responsible for its susceptibility to degradation when exposed to adverse factors (high temperatures, oxygen, light, low pH), which reduces its stability and causes color degradation, thus limiting its use in some processed foods (Montenegro et al., 2004).

The encapsulation was defined as a good alternative to improve the stability of bioactive pigments (Barbosa et al., 2005; Rascón et al., 2011; Ravichandran et al., 2014; Rocha et al., 2012; Silva et al., 2013; Tonon et al., 2010). It is a technology that allows covering solids, liquids or gasses in microscopic capsules, creating a physical protection barrier between the encapsulated compound (core) and the encapsulation material (wall) (Özkan and Bilek, 2014).

Numerous encapsulation techniques have been developed, among which spray-drying is the most commonly used and the cheapest in the food industry, allowing to transform liquid solutions into homogeneous powders efficiently. Approximately 90% of microencapsulated products are obtained by spray-drying (DJordjević et al., 2015). The process involves atomization of a liquid emulsion (core/wall material) in a hot chamber, where the water evaporates, and the dry microparticles are collected (Loksuwan, 2007).

Wall material is a determining factor in the microencapsulation efficiency and the stability of the obtained powders (Kandansamy and Somasundaram, 2012). Polysaccharides such as gum arabic (GA), maltodextrin (MD) with different dextrose (DE) equivalent, and some proteins are frequently used as encapsulation materials, either individually or in a blend (Barbosa et al., 2005; DJordjević et al., 2015; Özkan and Bilek, 2014).

With the aim of improving the stability of carotenoids, several studies have been carried out to obtain microcapsules of bixin (Barbosa et al., 2005; De Marco et al., 2013; Parize et al., 2008; Zhang & Zhong, 2013), curcumin (Cano-Higueta et al., 2015; Wang et al., 2009), saffron (Rajabi et al., 2015), astaxanthin (Bustos-Garza et al., 2013), paprika carotenoids (Rascón et al., 2011), and lycopene (Rocha et al., 2012). However, there is no available information yet about research to encapsulate norbixin by the spray-drying method.

The aim of this work was to study the spray-drying microencapsulation of norbixin using blends of MD and GA as wall materials at different MD:GA ratios, to evaluate the process efficiency and characterize obtained powders.

2 Materials and methods

2.1 Materials

The annatto seeds were acquired from the local market in Porto Alegre, RS, Brazil. The edible polysaccharides Gum Arabic SYNTH (Porto Alegre, RS, Brazil) and Maltodextrin DE20 Vallens (Porto Alegre, RS, Brazil) were used as wall materials.

2.2 Preparation of the bixin standard

The extraction of bixin was carried out according to Rios & Mercadante (2004). First, two extractions with 50 mL hexane were performed to remove lipids. The seeds were separated by filtration. Then, two extractions with 50 mL methanol were performed to remove polar compounds, and the seeds were filtered again. The bixin was immediately extracted with ethyl acetate by magnetic stirring for 15 min and the extracts concentrated in a rotary evaporator (Fisatom M802, São Paulo, Brazil). Finally, 10 mL of dichloromethane were added to the flask to recover the dry bixin; the container was placed in a cold bath, and 30 mL of ethanol (99.7%) were added slowly to it. The final extract was stored at -18°C for 12 hours to allow the formation of bixin crystals.

2.1 Preparation of the norbixin standard

The extract with high norbixin content was prepared through saponification of bixin as previously described by Rios & Mercadante (2004). Thus 50 mL of KOH (10%) methanolic solution were added to the bixin extract, and this was stored in the absence

of light at 25°C for 12 hours. Norbixin salts were recovered in separating funnel with 100 mL of ethyl acetate/ethyl ether and acetic acid until the extract reached a pH around 3.5. It was subsequently washed with water several times until reaching a pH of 6.0, and the norbixin extract was dried in a rotary evaporator (Fisatom M802, São Paulo, Brazil).

2.2 UV/Visible Spectrophotometric Analysis

First, the norbixin was dissolved in ethanol (99.7%), and an aliquot of the extract was diluted in a KOH (0.5%) aqueous solution that was used as a standard. Finally, the norbixin concentration was quantified with a Shimadzu SPECTROPHOTOMETER UV-1800 (Tokyo, Japan) at 453 nm. (Alves et al., 2006). A standard curve was developed for the quantification of norbixin in the range from 2.30 to 27.64 µg/mL with a correlation factor (R^2) de 0.99. For the calculations, the absorptivity coefficient ($E_{1\text{cm}}^{1\%} = 3473$) of the standard solution (Yabiku, & Takahashi, 1992) was considered along with the following equation:

$$x_{(\mu\text{g})} = \text{Abs} \times \gamma \times 10^6 / E \times 100 \quad (1)$$

Abs = Absorbance

γ = dilution (mL KOH 0.5%)

E = Absorptivity coefficient

2.3 Preparation of solutions for the drying process

Based on previous microencapsulation studies of bixin extracts performed by Barbosa et al. (2005) and De Marco et al. (2013) were tested formulations of wall materials with different %TSS and carried out analysis of viscosity with the aim to define core:wall material ratio and %TSS. Then to prepare each formulation 20 mg of norbixin crystals were diluted in ethanol to final volume of 100 mL with 20% total soluble solids (TSS) at the different MD:GA proportions (100:0, 85:15, 65:35, 50:50, 35:65, 15:85, 0:100). Each formulation was magnetically stirred at 50°C for 30 min, and all formulations were stored at -4°C.

Subsequently, after it was carried out the characterization of the microcapsules obtained and evaluated the efficiency of encapsulation for each formulation, based on these previous results an additional formulation was prepared using the proportion MD:GA (0:100), with 40% TSS and 30 mg of norbixin.

2.4 *Microencapsulation by spray-drying*

The drying process was carried out in a Mini Spray Dryer Büchi B-290 (Flawil, Switzerland). Were used a feeding flow of 0.39 L h⁻¹, inlet and outlet air temperatures were 150°C and 75°C, respectively, airflow rate 600 N L h⁻¹ and the spray nozzle diameter was 0.7 mm. The same parameters were used for all formulations, and each experiment was performed in duplicate.

2.5 *Process yield*

A gravimetric method was used to calculate the microencapsulation process yield (PY) (Fang and Bhandari, 2010), according to the following equation:

$$PY(\%) = (\text{Mass of MCN (g)}/\text{Mass of initial solids (g)}) \times 100 \quad (2)$$

2.6 *Microencapsulation efficiency*

In order to evaluate the microencapsulation efficiency (ME), the norbixin retained on the surface (NS) and inside (NR) of the MCNs was quantified according to the method described by Stoll et al., (2016) with some modifications. The exhaustive extraction of NS was followed with 1 mL of methanol/ethanol (1:1) to 100 mg MCN. The sample was mixed for 2 minutes at room temperature in a vortex, centrifuged (3000 x g, 10 min, 4°C), and the supernatant was collected and stored in amber flasks. Subsequently, to obtain the NR, 1 mL of ethanol/water (1:1) was added to the sediment, mixed for 2 minutes in Ultra-turrax (IKA T25, Taiwan, China) at room temperature, and then centrifuged (3000xg, 10 min, 4°C). The supernatant was collected and stored in amber flasks. Finally, each extract was analyzed by HPLC and quantified about the content norbixin, to calculate ME following the equation:

$$ME (\%) = (NR / (NR+NS)) \times 100 \quad (3)$$

2.7 *Moisture content*

MCNs were analyzed using a thermal scale OHAUS MB25 immediately after each experiment. A sample of 1 g was placed on the weighing pan, and the water was withdrawn by heating the sample with halogen light. After 60 seconds, the moisture content was obtained, expressed as a percentage.

2.8 Water Solubility Index (WSI)

The water solubility index (WSI) was defined as the degree to which the MCNs were solubilized in an aqueous medium, and it was determined according to a procedure described by Stoll et al. (2016). To each 2.5 g MCN were added 30 mL water and mixed for 2 min. in a digital Ultra-turrax (IKA T25, Taiwan, China). The mixture was taken to a water bath at 25°C for 30 min, centrifuged (3500 x g, 15 min, 4°C), and the supernatant was collected to dry in an oven at 105°C overnight. A number of solids in the supernatant was express in percentage to indicate the WSI.

2.9 Colorimetric Analysis

The color was measured using a colorimeter Chromameter CR-400 (Konica Minolta, Tokyo, Japan), with the parameters (L^* , a^* , b^*) according to the CIELAB system, where L^* is the lightness (0 = white) to darkness (100 = black) variation, a^* the variation between green ($<a^*$) and red ($>a^*$), and b^* the variation between blue ($<b^*$) and yellow ($>b^*$). A white ceramic disc was used for calibration before carrying out any measurements. The Hue angle and Chroma values, which indicate the color tone of the sample (0 or 360 = red, 90 = yellow, 180 = green and 270 = blue) and vividness of color saturation respectively, were calculated using the following equations:

$$\text{Hue} = \tan^{-1} (b^*/a^*) \quad (4)$$

$$\text{Chroma} = (a^*)^2 + (b^*)^2 \quad (5)$$

2.10 Particle size distribution

Particle size distribution was measured with a Mastersizer 2000 (Malvern Instruments Ltd., Worcestershire, UK) to which a dry dispersion module Sirocco 2000 was attached, using the laser diffraction technique. The volume-weighted mean diameter expressed as a diameter $D[4,3]$, which is the weighted average volume diameter, assuming spherical particles with the same volume as the actual particles. The particle size distribution was calculated by the equation:

$$\text{Span} = (d(0.9) - d(0.1))/d(0.5) \quad (6)$$

Where $d_{0.9}$, $d_{0.1}$, and $d_{0.5}$ are the diameters at 90, 10, and 50% of the cumulative size distribution curve, respectively.

2.11 Scanning electron microscopy (SEM)

The morphology of microparticles was evaluated by SEM using an equipment Zeiss EVO MA 10 (Carl Zeiss, Berlin, Germany). Firstly, the MCNs were attached in stubs with a two-sided metallic adhesive tape. The stubs were coated with a fine layer of gold and examined at 2.50 kx, 10.00 kx and 20.00 kx magnifications. The acceleration voltage was 5.00 kV and the images were captured and recorded.

2.12 High-performance liquid chromatography (HPLC)

Analyses for norbixin quantification were performed according to Montenegro et al. (2004) using an HPLC (Agilent series 1100, Santa Clara, CA, USA) equipped with an online degasser, a quaternary pump, a UV-visible detector and an automatic injector. The separation of pigment was conducted on a column C18 Spherisorb ODS-2 (150 × 4.6 mm i.d.; 3 µm particle size), acetonitrile/acetic acid (2%) (63:35, v:v) as mobile phase at a flow rate of 1 mL/min and detection at 450 nm. The injection volume was 5 µL, and the column temperature was kept at 29°C. The CHEMSTATION® software was used to collect and process the data. For the injection, all samples were pre-filtered using a modified PTFE membrane for aqueous and organic solvents with a pore diameter of 0.45 µm (Millipore, SP, Brazil) and each injection was carried out in triplicate.

For quantification of norbixin was used a standard curve that was developed previously with different norbixin concentrations. The limits of detection and quantification values were 0.0304 µg/mg of microcapsules and 0.0305 µg/mg of microcapsules, respectively and were calculated according to the procedure described by Ertaş et al., (2007).

2.13 2,2'-Azino-Bis(3-Ethylbenzothiazoline-6-Sulfonic Acid) Radical Scavenging Activity

The antioxidant activity of norbixin presents in MCNs was evaluated after the spray-drying process, the extraction of norbixin was performed from MCNs following the procedure described by Stoll et al. (2016) with modifications. 2.5 g MCN (566 µg norbixin) were diluted in 2 mL distilled water, mixed in a vortex for 2 min and then centrifuged (3000xg, 10 min, 4°C). The supernatant was collected. For norbixin crystals were followed the same process.

Subsequently, the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) scavenging capacity of the MCN samples was determined according to a modified method previously described by Re et al. (1999). ABTS⁺ radical was prepared by reaction of 5 mL ABTS (7mM) with 88 μ L potassium persulfate (140 mM), both solutions previously prepared. The mixture was stored at room temperature in the dark for 16 hours before use. ABTS⁺ solution was diluted in ethanol until achieving an absorbance of 0.700 ± 0.020 at 734 nm (Spectrophotometer Shimadzu CPS-240A, Japan). Then, 100 μ L of norbixin extract was added to 1 mL of the diluted ABTS⁺, and the absorbance values were collected after 6 min. Each analysis was carried out in triplicate. A standard curve was developed with different concentrations of Trolox (11 to 176 μ M) and the results were expressed as μ mol TE/g dry sample.

2.14 Statistical Analysis

Results were expressed as mean value \pm standard deviation. One-way analyses of variance (ANOVA) and a multiple-range test were performed to correlate the data and to establish statistically significant differences ($p < 0.05$) with a 95% confidence interval. All calculations were performed with the STATGRAPHICS Centurion software.

3 Results and Discussion

3.1 Norbixin standard

Dry bixin crystal was saponified, and conversion of 100% to norbixin was achieved (100% recovery). Rios & Mercadante, (2004); Kovary et al., (2001); using the same methodology reported similar purity values to norbixin: 98% and 100%, respectively. These results indicate that the employed method permits to obtain an annatto extract with a higher degree of purity. Several studies were developed to extract annatto pigments trying to reach better degrees of recovery and high percentages of extraction yield by using other procedures, such as the ones reported by Alves et al. (2006) and Yolmeh et al. (2014), but does not exist information about conversion process to norbixin or purity degree.

3.2 Microencapsulation efficiency (ME) and process yield (PY)

During the drying process, norbixin purity was maintained; after HPLC analyses of norbixin crystals and MCNs, both achieved a 100 % of purity. The chromatograms

have not exhibited a formation of degradation compounds as isomers or unknown substances due to high temperatures and other parameters of equipment of spray-drying, which means the purity was not affected during the drying process. (Supplementary data)

Its important that in the microencapsulation process by spray-drying the encapsulation material remains inside of microcapsule and minimal quantities on the surface with the wall material. One main factor that influences in the microencapsulation efficiency is the high solubility of the core material and its low viscosity when used a high content of solids (Özkan and Bilek, 2014). The results, for this study, showed that ME depends mainly on the wall materials ratio MD:GA and their specific properties, on the total soluble solids (TSS) percentage, and on the core:wall material ratio. The ME value for each formulation MD:GA showed significant differences ($p < 0.05$). (Fig. 1.)

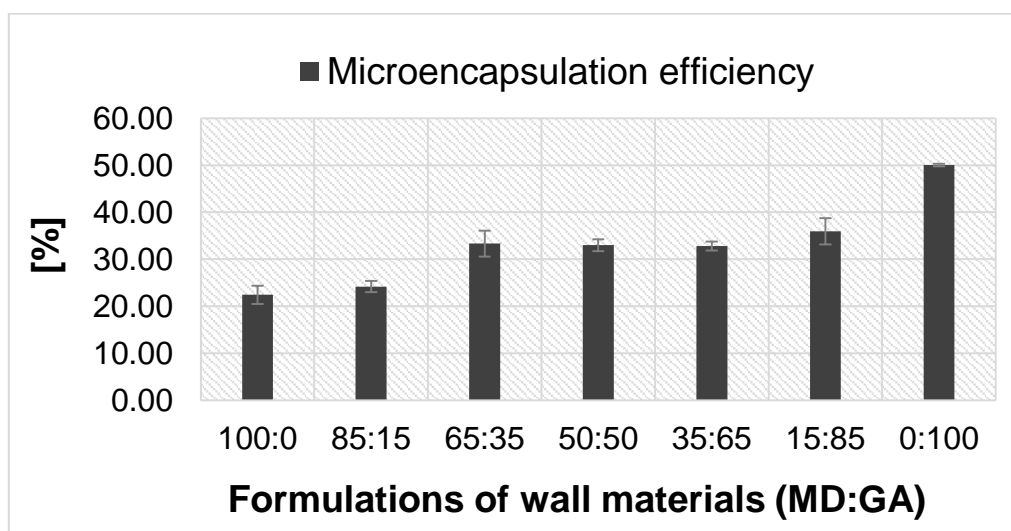


Fig.1. Process yield (PY) and microencapsulation efficiency (ME) as functions of the wall materials ratio maltodextrin (MD) and gum arabic (GA) in each formulation.

In consequence, ME is significantly affected by the addition of gum arabic, increasing from 22.52% (without of gum arabic) to 50.02% according to the proportion of wall materials. The increase of ME means a high amount of norbixin remains inside of the microcapsules. When GA was used in higher quantities than other components of blends, better ME was reached. Similar results were found in microencapsulation of bixin (Barbosa et al., 2005); curcumin (Cano-Higueta et al., 2015); flaxseed oil (Carneiro et al., 2013); betalains (Ravichandran et al., 2014); phenolic compounds (Nunes et al., 2015) and anthocyanins (Stoll et al., 2016).

Specific wall material properties, emulsion characteristics, operational parameters (inlet and outlet temperatures, feed flow, air flow and moisture, powder particle size, etc.), and the core to wall material ratio are factors with considerable influence on ME during microencapsulation of bioactive compounds (Mahdavi et al., 2016; Ersus and Yurdagel, 2007; Jafari et al., 2008; Saéñz et al., 2009; Saikia et al., 2015).

Properties of wall materials depend on their chemical structure. GA is formed by a ramified carbohydrate chain and a small amount of glycoproteins linked by covalent bonds. This allows GA to interact with both hydrophilic and hydrophobic sections of molecule, and therefore it is an excellent emulsifying agent in a wide pH range, it is compatible with a great variety of polymers, and it has a great film-forming capacity, which allows it to better retain the encapsulated molecule (Mahdavi et al., 2016; Krishnan et al., 2005). MD is hydrolyzed short chain starch that acts as a barrier against oxygen. However, when maltodextrin was used as wall material, the lowest efficiency was obtained, probably due to lack of emulsification and low film-forming capacity (Loksuwan, 2007). When the higher %TSS is used, results in a higher viscosity of the emulsion. Probably, this increases ME due to the reduction of time required for the formation of a semi-permeable membrane on particles surface during drying (Mahdavi et al., 2016). When the difference between the amounts of the core material and wall material is lower, consequently the ME is higher (De Marco et al., 2013; Saéñz et al., 2009; Saikia et al., 2015).

Process yield is a value determined by the efficiency to recover the powder from the system about the amount of total soluble solids in the feed. A high process yield allows to establish a cost-benefit relation that determines the viability of the spray-drying technique and a specific MD:GA ratio to encapsulate norbixin. In a similar way, PY shows a variation between 59.48% and 91.35% and it was significantly influenced by MD:GA ratio and %TSS. Rajabi et al. (2015), reported that high process yield was obtained for the encapsulation of bioactive compounds in absence or presence of minimal quantities of GA when high %TSS was used. The formulation that achieved the highest PY was 50:50 (MD:GA), however, the ME was less than 50%. In the absence of MD, a PY larger than 60% and the best ME ($p < 0.05$) were obtained. The same effect of GA was reported by Di Battista et al. (2015), who used a higher GA amount about MD and 20-25% TSS.

Powder loss was mainly caused by adherence of semi-moist drops to the chamber walls, adherence of dry powder to cyclone filter walls, and the recovery difficulty

of cyclone filter. Fernandes et al. (2012), reported that at low inlet temperatures (140 - 150°C) a moist product was obtained and it tended to adhere to the drying chamber walls and cyclone filter wall. However, Rascón et al. (2011), reported that the use of GA and a higher inlet temperature increased PY and ME for the microencapsulation of paprika oleoresin. A very low ME can be obtained by using a wall material of low emulsification and high inlet temperatures to encapsulate sensitive carotenoids such as lycopene (Rocha et al., 2012). Another factor that could have an influence on PY is the core to wall material ratio when the relation between these materials was lower; higher PY was achieved (De Marco et al., 2013; Saénz et al., 2009; Saikia et al., 2015).

Based on these results, an additional spray-drying experiment was performed with 0:100 (MD:GA), 40% SS and 30 mg norbixin to optimize ME. The operational parameters were kept constant, and an ME of 75% was achieved, the final concentration of norbixin was 226.40 µg/g.

3.3 Particle characterization

The results of moisture content, solubility, volume-weighted mean diameter $D_{4,3}$, and Span value of powders produced with different wall materials are presented in Table 1.

Table 1. Characterization of particles in formulations with different proportions of wall materials.

Formulation MD:GA	Moisture (%)	Particle size distribution		Water solubility index (%)
		$D_{4,3}$ (µm)	SPAN	
100:0	1.50 ± 0.00 ^d	5.02 ± 0.06 ^a	1.63 ± 0.13 ^{ab}	95.58 ± 0.99 ^a
85:15	3.50 ± 0.00 ^a	5.07 ± 0.55 ^a	1.69 ± 0.07 ^a	92.13 ± 3.21 ^b
65:35	2.75 ± 0.35 ^b	5.20 ± 0.11 ^a	1.34 ± 0.07 ^c	91.04 ± 0.80 ^{bc}
50:50	3.50 ± 0.00 ^a	5.23 ± 0.15 ^a	1.52 ± 0.10 ^b	90.94 ± 0.73 ^{bc}
35:65	3.50 ± 0.00 ^a	5.26 ± 0.32 ^a	1.60 ± 0.03 ^{ab}	89.51 ± 2.13 ^c
15:85	2.00 ± 0.00 ^c	5.42 ± 0.29 ^a	1.61 ± 0.02 ^{ab}	91.32 ± 0.74 ^{bc}
0:100	3.25 ± 0.35 ^a	5.03 ± 0.58 ^a	1.71 ± 0.12 ^a	91.46 ± 1.33 ^b

Different letters in the same column indicate statistically significant differences between samples at $p < 0.05$. (MD: Maltodextrin, GA: Gum Arabic, SPAN: Particle size distribution)

3.3.1 *Moisture content*

The moisture content of powders had a variation between 1.50 and 3.50%. Pang et al. (2014), reported that inlet drying temperature, %TSS in each solution, and specific hygroscopicity of each wall material, are all factors that influence the moisture content. However, operational parameters were kept constant for all experiments in this study, making it most likely for environmental factors to be responsible for the variations in moisture content, since it was not possible to keep the relative humidity constant in the laboratory for all the experiments.

Although moisture content values were statistically different for all formulations, the average value achieved was very low. Therefore it was considered as an optimal value to maintain quality and prevent degradation of the MCNs.

3.3.2 *Particle size distribution and morphology*

In general, samples did not show significant differences in diameter when different wall materials were used. The average diameter was $5.018 \pm 0.14 \mu\text{m}$, which means all obtained powders were microparticles. The Fig. 2. shows the particle size distribution for the formulations 0:100; 50:50 and 100:0 (MD:GA) that showed significant differences ($p < 0.05$) when was evaluated the process yield (PY). Diameter does not depend on type or proportion of wall materials, it is influenced by the inlet temperature, feed flow, and %TSS since heat transfer coefficient is higher at higher temperatures, and thus a faster water evaporation is achieved (Janiszewska and Włodarczyk, 2013). Therefore, a higher inlet temperature permits to obtain the lower particle diameter. However, the microcapsules showed a significant variation in Span according to the type of wall material. Span values ranged from 1.34 (65:35, MD:GA) to 1.71 (0:100, MD:GA), being gum arabic the material that resulted in the highest particle size distribution. The difference could be related to the viscosity of each formulation, since the more viscous the solution, the less homogeneous the particle sizes after drying (Tonon et al., 2011). The advantage of obtaining powders with a Span value < 2 is related to solubility. With a low Span value, homogeneity is higher (Fernandes et al., 2014), because there are no significant changes in the physical properties of the powders during drying. Therefore their solubility improves.

For morphology, MCNs did not show differences when different wall materials were used, but the results exhibited differences in size and shape when %TSS was increased. Previously was obtained microcapsules of GA without norbixin (MC-empty).

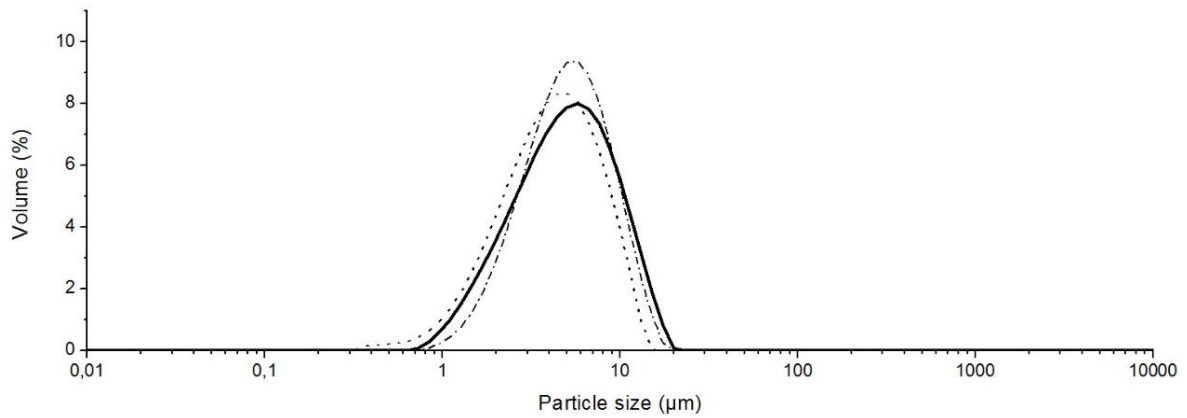


Fig. 2. Particle size distribution of formulations MD:GA 100:0 (···), 50:50 (-·-), 0:100 (-).

Figure 3. shows the images of SEM analyses that were carried out with samples: MC-empty, MCN 0:100 (MD:GA) - 20%TSS and 0:100 (MD:GA) - 40%TSS.

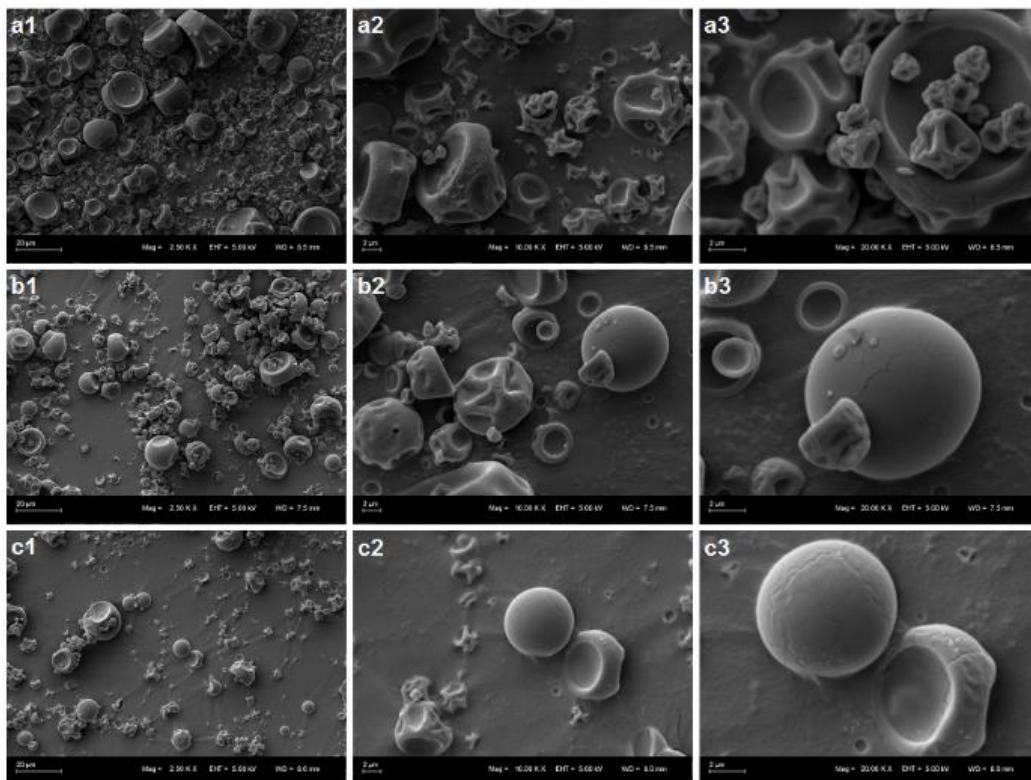


Fig. 3. SEM micrographs of the microcapsules of norbixin in different magnifications and composition: a) MC – empty 0:100 MD:GA; b) MD:GA 0:100 – 20%TSS; c) MD:GA 0:100 – 40%TSS; 1) 2.50 kx; 2) 10.00 kx; 3) 20.00 kx.

The morphology irregularities on the surface of microcapsules could be attributed to the faster evaporation of liquid droplets during the drying process that causes a constriction of microparticles (Loksuwan, 2007; Rocha et al., 2012). When the microcapsule of GA was empty it showed more cavities on the surface (Fig. 3.a), the same effect was founded by Di Battista et al. (2015). The morphology of MCNs 0:100 MD:GA (20 and 40 %TSS) was more spherical (Fig. 3.b,c), with a less formation of teeth concavities, similar images was reported by Bustos-Garza et al. (2013) for encapsulated astaxanthin with GA.

3.3.3 Water solubility index (WSI)

The WSI was >90 for all experiments, meaning that the microencapsulation process improves the solubility (Sousdaleff et al., 2013), but showed significant differences between formulations ($p<0.05$). The values were different for formulations 100:0 and 0:100 (MD:GA), because each wall material has a specific solubility, and the highest WSI was achieved when maltodextrin was used as the only wall material. The mixture of both wall materials causes a decrease in the solubility because GA has a lower WSI and needs high temperatures for achieves a better solubilization. The same tendency was observed by Loksuwan (2007) and De Marco et al. (2013), to encapsulate β -Carotene and bixin respectively, showing that WSI does not depend on the nature of the core material (hydrophilic or hydrophobic). It can be concluded that WSI depends on of the specific properties of wall materials that were used (Barbosa et al., 2005; Stoll et al., 2016).

3.3.4 Colorimetry of powders

The MCN obtained after each treatment showed significant differences ($p<0.05$) in the parameters L^* , a^* , and b^* as shown in Table 2. In all of the powders, the characteristic coloration of norbixin was observed, which means that the pigment was incorporated into the matrix of the materials used for microencapsulation (Bustos-Garza et al., 2013). High values of parameters a^* and b^* indicate a yellow or orange color, while the L^* value corresponds to a higher or lower brightness. L^* value of each wall material influences the luminosity of the powders because of its high proportion of the core material. The results for L^* showed statistically significant differences ($p<0.05$) because MD is lighter than GA, when wall materials were assessed on separate without norbixin the L^* value was 54.85 ± 0.10 and 52.51 ± 0.07 ; respectively. Therefore, each blend has a different luminosity.

Table 2. Color of powders microencapsulated with the different formulations of maltodextrin and gum arabic (MD:GA)

Formulation MD:GA	L^*	a^*	b^*	Chroma	Hue
100:0	48.90 ± 0.92 ^a	7.88 ± 0.06 ^b	17.55 ± 1.01 ^{cd}	19.24 ± 0.82 ^{bc}	71.63 ± 3.89 ^c
85:15	49.45 ± 0.36 ^a	5.58 ± 0.55 ^e	17.05 ± 0.52 ^{cd}	17.94 ± 0.61 ^{de}	90.96 ± 3.30 ^a
65:35	47.70 ± 0.81 ^b	7.00 ± 0.11 ^c	18.90 ± 1.83 ^{ab}	20.16 ± 1.60 ^b	83.55 ± 4.51 ^b
50:50	45.77 ± 0.57 ^c	9.74 ± 0.15 ^a	19.11 ± 0.66 ^a	21.45 ± 0.59 ^a	62.89 ± 1.43 ^d
35:65	46.24 ± 0.65 ^c	6.30 ± 0.32 ^d	17.84 ± 0.12 ^{bc}	18.92 ± 0.02 ^{cd}	86.64 ± 2.97 ^{ab}
15:85	48.00 ± 0.52 ^b	4.12 ± 0.29 ^f	16.60 ± 0.75 ^{ad}	17.50 ± 0.36 ^e	89.61 ± 0.88 ^a
0:100	46.83 ± 0.37 ^c	6.43 ± 0.58 ^d	14.90 ± 0.79 ^e	16.25 ± 0.45 ^f	74.22 ± 8.51 ^c

Different letters in the same column indicate statistically significant differences between samples at $p < 0.05$. (MD: Maltodextrin, GA: Gum Arabic).

MCNs with 50:50 (MD:GA) showed the highest a^* and b^* values, which indicate a dark orange color (Fig 4. b) according to CIELab color chart, because the greater amount of pigment was retained on the surface and less than 33.05% was kept inside of the capsule.

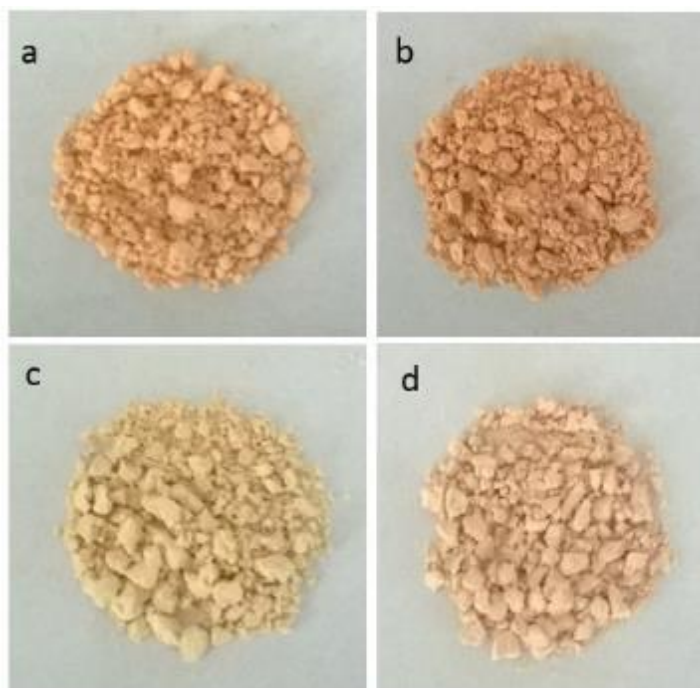


Fig. 4. Images of microcapsules obtained with formulations MD:GA, 100:0 (a), 50:50 (b), 15:85 (c), 0:100 (d).

MCNs with 15:85 (MD:GA) showed an ME of 36.00% and lower a^* and b^* values, indicating a light orange color due to the influence of the wall material in higher quantity (GA) (Fig 4. c). The same effect was found for MCNs with 100:0 and 0:100 (MD:GA), with ME of 22.52% and 50.02% respectively (Fig 4.a,d). The results showed that ME and wall materials ratio (MD:GA) have a dominant effect on the color of the microcapsules.

The MCNs with different formulations showed statistically significant differences in Hue angle values ($p < 0.05$) between 62.89 and 90.96°, all in the first quadrant of the CIELab color chart, indicating a variation between orange and yellow tones, and with different intensities due to the differences in the Chroma factor ($p < 0.05$) (Table 2). A direct relation of MCNs coloration with ME and MD:GA ratio was ratified. Still, all microcapsules were in the expected color range. The same effect was reported by Bustos-Garza et al. (2013) for microencapsulation of astaxanthin.

3.3.5 Antioxidant activity

Antioxidant activity of MCNs depends on the amount of norbixin retained inside of microcapsules, which means it has a direct relation to ME. Other studies about microencapsulation of natural colorings with different wall materials found that the powders with high ME showed good antioxidant activity as a consequence of this, especially when GA was used in major ratio than MD (Stoll et al., 2016; Tonon et al., 2010). Therefore, the antioxidant activity of the MCNs with the formulation that led to an ME of 75% was evaluated in this study, also previously was obtained microcapsules of GA without norbixin (MC-empty) and was analyzed its ABTS radical scavenging activity. The resulting value for MCN and MC-empty was $77.77 \pm 0.59 \mu\text{mol TE/g dry sample}$ and $13.49 \pm 0,64 \mu\text{mol TE/g dry sample}$, respectively. By these results, it could be inferred that approximately 64 $\mu\text{mol TE/g dry sample}$ is related to the norbixin fraction. Therefore, the antioxidant activity that was showed for MCN depends on norbixin, because this value for MC-empty was much lower when compared to the value of MCN. The antioxidant activity of GA may be caused by the protein fraction in the chemical structure of GA (Rodrigues et al., 2012; Tonon et al., 2010). The result of ABTS for MCN was almost 50 times lower than the value of ascorbic acid ($4276 \pm 85.19 \mu\text{mol TE/g dry sample}$). Nonetheless, more of 80% antioxidant activity was retained in MCN when was compared to the value of norbixin crystals ($71.92 \pm 3.77 \mu\text{mol TE/g dry sample}$), which means that antioxidant activity is not affected significantly by the drying process.

4 Conclusion

The norbixin crystals can be encapsulated with blends of MD and GA at different ratios as wall materials by spray-drying. It was found that the use of GA as the only wall material led to a high microencapsulation efficiency ($\approx 75\%$) and an acceptable process yield ($>60\%$). The high microencapsulation efficiency resulted in a high antioxidant activity for the norbixin microcapsules. Thus a water-soluble natural coloring with antioxidant properties and excellent solubility was obtained. Norbixin microcapsules are a promising ingredient for processed beverages, for which their stability under different adverse factors that define their future applications must be evaluated.

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SUPPLEMENTARY DATA

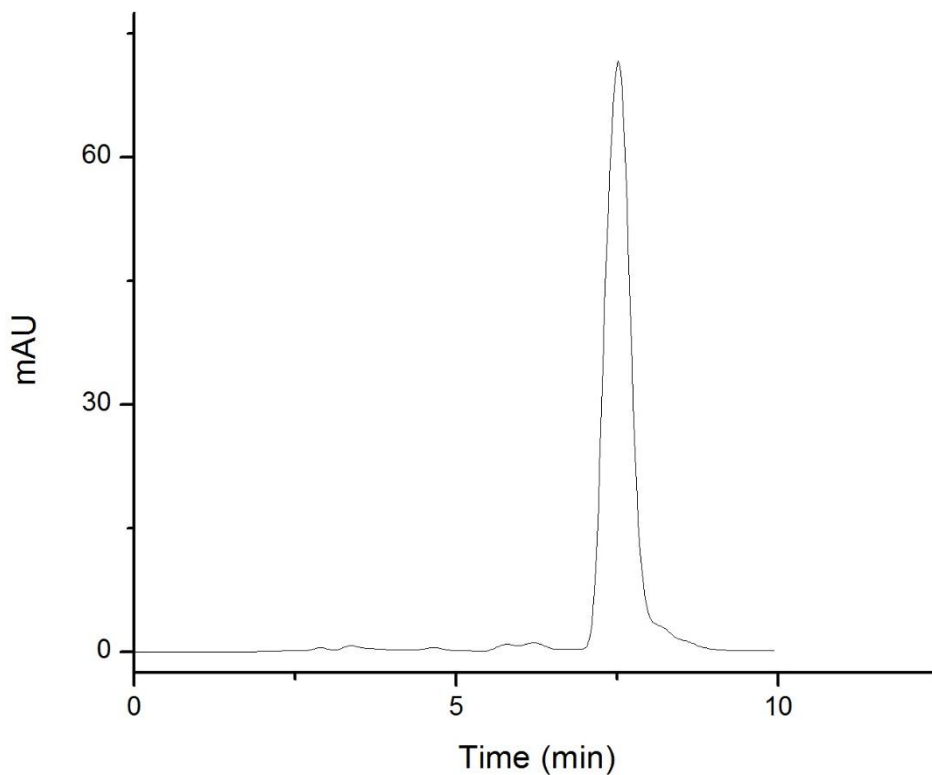


Fig. A1. HPLC chromatogram of norbixin crystals

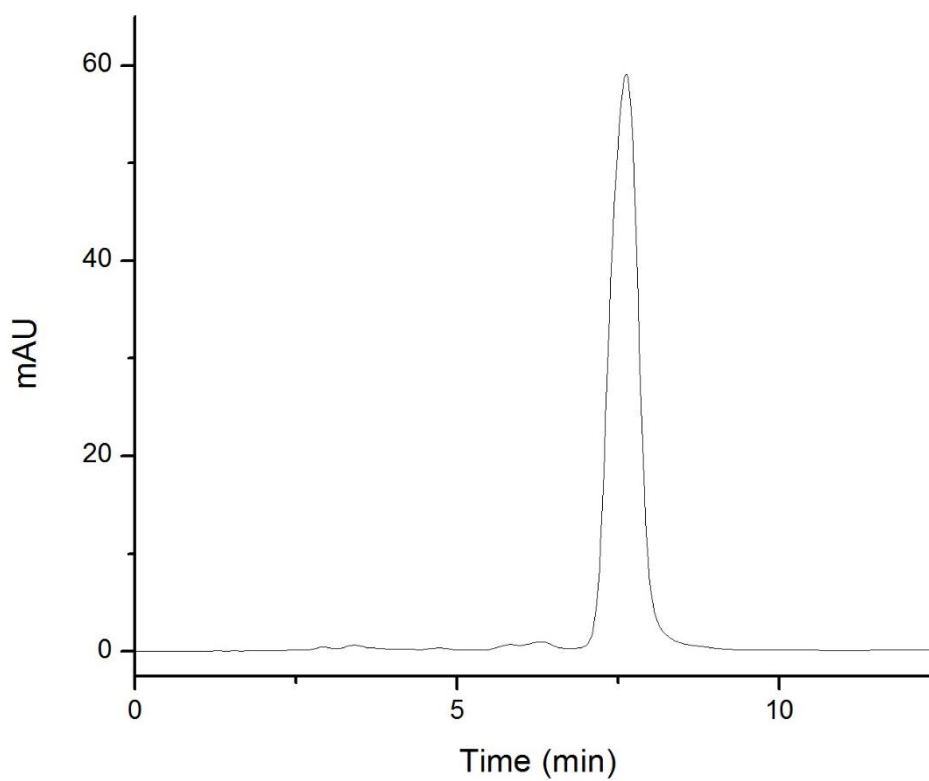


Fig. A2. HPLC chromatogram of norbixin microcapsules

ARTIGO 2

**THERMAL STABILITY OF NORBIXIN MICROCAPSULES OBTAINED BY
SPRAY-DRYING**

Artigo formatado de acordo com as normas da revista "Food and Bioprocess
Technology"

Thermal stability of norbixin microcapsules obtained by spray-drying

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Simone Hickmann Flôres, Alessandro de Oliveira Rios

ABSTRACT

Norbixin crystals were obtained from annatto seeds (*Bixa orellana* L.) and encapsulated by spray-drying using gum arabic as wall material. Norbixin microcapsules (MIC-Norb) showed good homogeneity, high solubility and a high microencapsulation efficiency (75% - 480.25 ± 2.33 $\mu\text{g/g}$ microcapsules). The study of stability was carried out in aqueous model system at temperatures of 60, 90 and 98°C for 300 min. Thermal degradation kinetics in aqueous model systems followed a first order kinetic reaction. The activation energy (E_a) required for degradation was $E_a = 15.08$ kcal/mol, higher that required for free norbixin crystals ($E_a = 7.61$ kcal/mol). MIC-Norb showed a high thermal stability with longer shelf life. Thermal degradation depended directly from temperature and heating time and the results may indicate the stability of the encapsulated pigment in aqueous food systems.

Keywords: annatto; microencapsulation; thermal stability; gum arabic; norbixin

1 Introduction

Norbixin (9'-*cis*-6,6'-diapocarotene-6,6'-dioic acid), is a carotenoid with *cis* configuration and one of the pigments found on the surface of annatto seeds (*Bixa orellana* L.). It occurs in nature in small amounts but it can be obtained through methods based on saponification of bixin extracted from annatto seeds. Bixin is a natural food coloring soluble in fats but insoluble in water. Upon to exposure to alkaline solutions, the methylester is hydrolyzed to obtain a hydro-soluble salt of norbixin (Scotter 2009; Van Chuyen et al. 2012) of which solvents used for the extraction are completely removed to obtain purified norbixin crystals (Rios and Mercadante 2004).

Besides colorification capacity, the most important attribute of norbixin is its antioxidant activity (Cardarelli et al. 2008; Kiokias and Gordon 2003). Several studies have proven that annatto extracts (bixin and norbixin) act effectively on deactivation of reactive oxygen species (ROS) that can cause DNA damages (Júnior et al. 2012; Kovary et al. 2001; Rios et al. 2009; Rios et al. 2007).

Norbixin is used as a natural dye in processed foods, since it has no adverse toxicological effects on humans when ingested in minimal quantities on a daily intake (Hagiwara et al. 2003). The color of norbixin extracts can be in the orange-yellow-red range according to the final concentration of the compound (Satyanarayana et al. 2010). They are used as food additives in butter, cheese, bakery products, ice cream, sausages, extruded products, soft drinks, sodas and liquors, and as an advantage, have lower costs than other natural dyes (Cardarelli et al. 2008). As all carotenoids, norbixin is susceptible to degradation when exposed to high temperatures, light and oxygen, which limits its applications in food industry. It is also hydro-soluble because of its chemical structure, but it has a low solubility, making it necessary to develop methods to obtain formulations suitable for beverages.

Spray-drying microencapsulation techniques for natural pigments is an effective way of improving stability and solubility of compounds, causing a better conservation and an increase in shelf life (Özkan and Bilek 2014; Rocha et al. 2012). In the food industry this technique is widely used because it is simple and transforms liquids into solids being necessary to use only small percentages of total soluble solids (Bustos-Garza et al. 2013). It is defined as a drying process in which a compound of interest (core material) is homogenized with polysaccharides, proteins or lipids (wall materials) in an aqueous solution in order to be sprayed. Because of high temperatures, water evaporates from the liquid drops and a polymeric film is formed around the core, acting as a protection barrier (Kandansamy and Somasundaram 2012), and after the encapsulated microparticles are collected separately.

Wall material polymers must have certain properties, being the most important an emulsifying ability, film-forming capacity and low viscosity at high temperatures. Gum Arabic is most commonly used because it gathers all the desired conditions and shows a high encapsulation efficiency and help to improve stability, as proven in other microencapsulation studies for natural dyes: bixin (Barbosa et al. 2005); curcumin (Cano-Higuera et al. 2015); betalains (Ravichandran et al. 2014) and anthocyanins (Stoll et al. 2016).

Several studies have been performed with the aim of evaluating norbixin incorporation to different foods, such as extruded corn snack products (Rios and Mercadante 2004); and other works have also evaluated its light and thermal stability applied to fruit juices and dairy products during storage (Parvin et al. 2011; Prabhakara Rao et al. 2002). Kinetic degradation of bixin, both free and encapsulated in model

aqueous systems, was studied by Rios et al. (2005); Silva et al. (2007) and Lobato et al. (2015), respectively. Ferreira et al. (1999), carried out a study on thermal degradation of commercial norbixin solutions, however, there is no available information about the evaluation of thermal stability for microcapsules of norbixin.

The aim of this study was to evaluate the kinetics of thermal degradation in model aqueous systems for norbixin microcapsules obtained by spray-drying with gum arabic as wall material, this in order to define an estimated shelf life for applications as natural dye in processed foods.

2 Materials and methods

Food grade polysaccharide Gum Arabic SYNTH (Porto Alegre, RS, Brazil) was used as carrier agent. The annatto seeds were acquired from the local market in Porto Alegre, RS, Brazil. All reactants were of analytical grade and suitable for HPLC.

2.1 Preparation of the norbixin standard

Purified norbixin extract was obtained through the procedure described by Rios and Mercadante (2004). First, bixin was washed and extracted from annatto seeds with organic solvents, and the extract was dried in a Fisatom rotary evaporator. Then, the extract was dissolved in dichloromethane and absolute ethanol was added for the formation of bixin crystals. Finally, saponification of bixin crystals was carried out using a methanolic KOH solution (10%), the obtained norbixin salt was separated from the extract using a separation funnel and the obtained norbixin extract was dried in a Fisatom rotary evaporator and stored at -18°C.

Purity of the norbixin crystals was evaluated by High Performance Liquid Chromatography (HPLC).

2.2 Quantification of norbixin

Concentration of norbixin diluted in ethanol (99.7%) was quantified by spectrophotometry using an UV Spectrophotometer UV-1800 Shimadzu at 453 nm (Alves et al. 2006) and absorptivity value $E_{1\text{cm}}^{1\%} = 3473$ (Yabiku and Takahashi 1992).

2.3 Microencapsulation by Spray drying

A formulation was performed by dissolution of 40 g Gum Arabic (GA) in 80 mL distilled water and added 30 mg norbixin crystals diluted in 20 mL ethanol (99.7%). The final solution was magnetically stirred at 40°C for 30 min. A homogenous emulsion was obtained and it was stored at -4°C.

Microencapsulation process was carried out in a Mini Spray Dryer Büchi B-290. Operational parameters were 6.6 mL/min feed flow and 150°C inlet temperature. The experiment was performed in duplicate. The obtained microcapsules were immediately vacuum packed in plastic bags and stored at -4°C. An additional formulation was prepared without norbixin crystals and the spray-drying process was carried out with the same parameters to obtain coreless microcapsules (MIC-Empty).

2.4 Characterization of microcapsules

The moisture of norbixin microcapsules (MIC-Norb) was measured with a thermal OHAUS MB25 after the spray drying experiment.

The mean volume-weighted diameter was measured with a Mastersizer 2000 (Malvern Instruments Ltd., Worcestershire, UK) coupled to a dry dispersion module Sirocco 2000, based on the laser diffraction technique. The average was expressed as a diameter $D_{(4.3)}$. For the calculation of particle size distribution (*Span*), the following equation was used:

$$\text{Span} = (d(0.9) - d(0.1))/d(0.5) \quad (1)$$

Where $d_{0.9}$, $d_{0.1}$, and $d_{0.5}$ are the diameters at 90%, 10%, and 50% of the cumulative size distribution curve, respectively (Fernandes et al. 2014).

To evaluate the morphology of the microcapsules the samples were placed on stubs, fixed with double-faced carbon tape, and sputter-coated with a 15 – 20 nm layer of gold. The samples were analyzed by scanning electronic microscopy (SEM) with an equipment EVO MA 10 (Carl Zeiss, Germany) using an acceleration voltage of 5 kV at different magnifications (2.50 kx, 10.00 kx and 20.00 kx).

A determination of water solubility index (WSI) was carried out according to a procedure described by Stoll et al. (2016). Firstly, 2.5 g MIC-Norb were dissolved in 30

mL water and mixed for 2 min using a digital Ultra-turrax (IKA T25, China). The mixture was maintained to a water bath at 25°C for 30 min, and finally centrifuged (3500 x g, 15 min, 4°C). Finally was collected the supernatant and was leaved in an oven at 105°C overnight. To express the WSI, the amount of dried solids was quantified and expressed in percentage.

Microencapsulation efficiency (ME) was evaluated with the method described by Stoll et al. (2016), with modifications. To extract the norbixin retained on the surface (NS), 100 mg MIC-Norb were taken to a 15 mL centrifuge tube with a mixture methanol:ethanol (1:1, v/v), this was stirred in a vortex and then centrifuged. The supernatant was collected and stored in amber flasks. Subsequently, a mixture ethanol:water (1:1, v/v) was added, it was stirred for 2 min in a digital Ultra-turrax (IKA T25, China) and then centrifuged to extract the norbixin retained inside of the microcapsule (NR). The supernatant was collected and stored in amber flasks. Stirring was performed for 2 min each time at room temperature and centrifuged, for centrifugation was used parameters (3000xg, 10 min, 4°C) in all experiments, which were performed in triplicate. The addition of NS and NR was considered the total norbixin concentration (NT). The concentration of MIC-Norb was measured by HPLC for all the extracts and ME was calculated using the Eq. (1).

$$ME (\%) = (NR/NT) \times 100 \quad (2)$$

2.5 Heating in a model system

Heating experiments were carried out in a model aqueous system. Each 100 mg MIC-Norb were taken to a 15 mL centrifuge tube with screw cap and the tube was then heated in a hot bath of water to 60, 90 and 98°C. A sample was periodically taken from the tube and put immediately into an ice bath. Ethanol:water (1:1, v/v) was added to the tube to extract norbixin from MIC-Norb and it was stirred for 2 min in a digital Ultra-turrax IKA T25. It was then centrifuged (3000xg, 10 min, 4°C) and the supernatant was collected. For the experiments with free norbixin (F-Norb), purified norbixin crystals were solved in an aqueous 0.5% KOH solution until reaching a concentration of 4.50 µg/mL, then 1mL aliquot was taken to a 10 mL amber flask and submitted to the same heating procedure as MIC-Norb. Norbixin concentration in MIC-Norb and F-Norb samples was measured by HPLC at 0, 15, 30, 45, 60, 90, 120, 150, 180, 240 and 300 min during the experiments. Kinetic parameters were calculated with the norbixin content results using

the software Origin 8.0 (Origin lab Co., MA, USA). Half-life time ($t_{1/2}$) was calculated following the Eq. (2) (Bustos-Garza et al. 2013):

$$t_{(1/2)} = \ln 2/k_t \quad (3)$$

Where: $t_{1/2}$ = Half-time value; k_t = Kinetic constant

2.6 Analysis of high performance liquid chromatography (HPLC)

HPLC (Agilent series 1100, Santa Clara, CA, USA) was used to quantify the norbixin concentrations and the analyses were carried out according to the method described by Montenegro et al. (2004). The equipment was used with an online degasser, a quaternary pump and an automatic injector. A column C18 Spherisorb ODS-2 was used and acetonitrile/acetic acid (2%) (63:35, v/v) was the mobile phase and elution was isocratic in a 1 mL/min flow rate. Norbixin was detected at 450 nm through an UV-visible detector coupled to the system and the CHEMSTATION® software was used for data processing. Before injection, the samples were filtered using a modified PTFE membrane for aqueous and organic solvents with a pore diameter of 0.45 μm (Millipore, SP, Brazil). All solvents were previously filtered using a Millipore vacuum filtration system with a 0.22 μm membrane (Millipore, SP, Brazil). All analyses were performed in triplicate.

A calibration curve was obtained to quantify the total norbixin concentration. The curve had a coefficient of determination (R^2) of 0.99. Solutions with different norbixin concentrations (2.30 to 27.64 $\mu\text{g/mL}$) were analyzed by HPLC and the areas obtained for each solution were used to create the curve. Limits of detection and quantification were calculated following the procedure described by Ertaş et al. (2007) and the values were 0.0304 $\mu\text{g/mg}$ and 0.0305 $\mu\text{g/mg}$, respectively.

3 Results and Discussion

3.1 Characterization of microcapsules

MIC-Norb obtained by spray-drying in this research showed a low moisture value of $3.00 \pm 0.50\%$. The final moisture content is a very important parameter that could affect significantly the quality of powders; low moisture prevents its fast deterioration during storage. Particle size distribution was monomodal with a Span of 1.96 ± 0.25 and

the volume-weighted mean diameter $D_{(4,3)}$ was $8.50 \pm 1.33 \mu\text{m}$; for MIC-empty the $D_{(4,3)}$ was $8.42 \pm 0.25 \mu\text{m}$ with a Span of 1.88 ± 0.06 . These results show that microcapsules with high size homogeneity (Span < 2) (Fernandes et al., 2014), and without significant differences in diameter of microcapsules in the absence of norbixin that also were obtained during the drying process (Fig. 1).

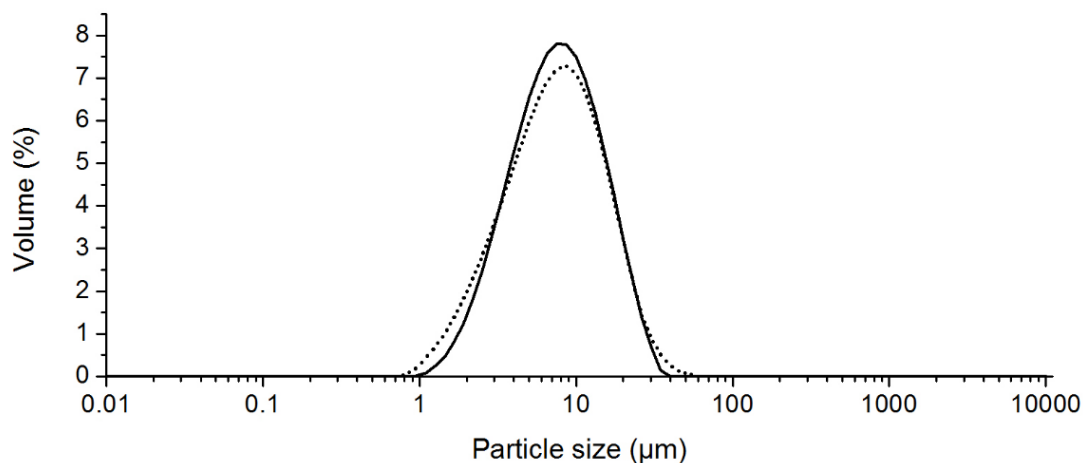


Fig. 1 Particle size distribution of MIC-Norb (···) and MIC-empty (—)

Differences in diameters are influenced by the properties of each material, percentage of total soluble solids (TSS), viscosity of formulation and spray dryer operational parameters (Jafari et al. 2008). GA has high viscosity, which produces particles with larger diameters and in turn better efficiency (Fernandes et al. 2014). According to SEM micrographs (Fig. 2), the microcapsules showed a near spherical form, typical of spray-dried particles. Cavity formation on the surface could be owing to the fast water evaporation, which causes shrinking of the particles by release of small quantities of air that were trapped inside of the microcapsules during the spray-drying process.

The same effect was reported in several studies that were carried out for study the microencapsulation of bioactive compounds when the GA was used as encapsulating material. (Barbosa et al. 2005; Bustos-Garza et al. 2013; Fernandes et al. 2014; Kanakdande et al. 2007). MIC-Norb had a high WSI ($91.46 \pm 1.33\%$) as consequence of successful encapsulation process, proving that the spray-drying technique allows to improve the solubility of the compounds (Wang et al. 2009).

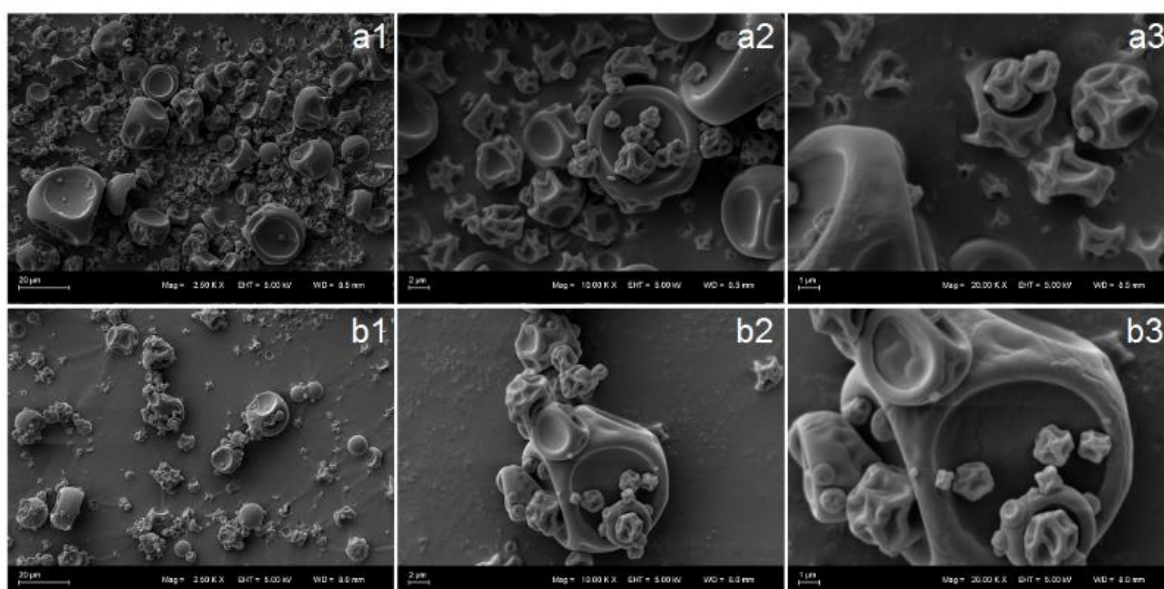


Fig. 2. SEM micrographs of the microcapsules of norbixin in different magnifications and composition: a) MC – empty; b) MIC-Norb; 1) 2.50 kx; 2) 10.00 kx; 3) 20.00 kx.

The process allowed achieving high norbixin retention inside of microcapsules, resulting in high ME (75%), corresponding to $480.25 \pm 2.33 \mu\text{g/g}$ of microcapsules. Other studies on spray-drying carotenoids microencapsulation with GA also reached high efficiency percentages, such as bixin (Barbosa et al. 2005) or curcumin (Cano-Higueta et al. 2015).

Through norbixin content quantification by HPLC analyses of norbixin crystals and MIC-Norb was evaluated the purity of norbixin extracts, both achieved a 100 % of purity. The chromatograms was not exhibited a formation of degradation compounds as an isomers or unknown substances, that means the purity was not affected during the spray-drying process. (Supplementary data)

3.2 Heating in a model system

Heating experiments were previously performed with F-Norb at 60 and 75°C for 240 min. A low decrease of the norbixin content was observed and this decrease continued constant from 90 min. Then, temperatures 60, 90 and 98°C were chosen and the heating was performed for 300 min. The results of norbixin concentration obtained by HPLC analysis were used to obtain a degradation curves for each temperature. The temperature increase results in a compound loss over time, and such effect was observed for both, F-Norb and MIC-Norb (Fig. 3). All temperatures showed first order kinetics and kinetic degradation constants (k) were calculated through an exponential

regression fit for F-Norb and MIC-Norb with a correlation factor of ($0.90 < R^2 < 0.96$) and ($0.90 < R^2 < 0.98$) respectively.

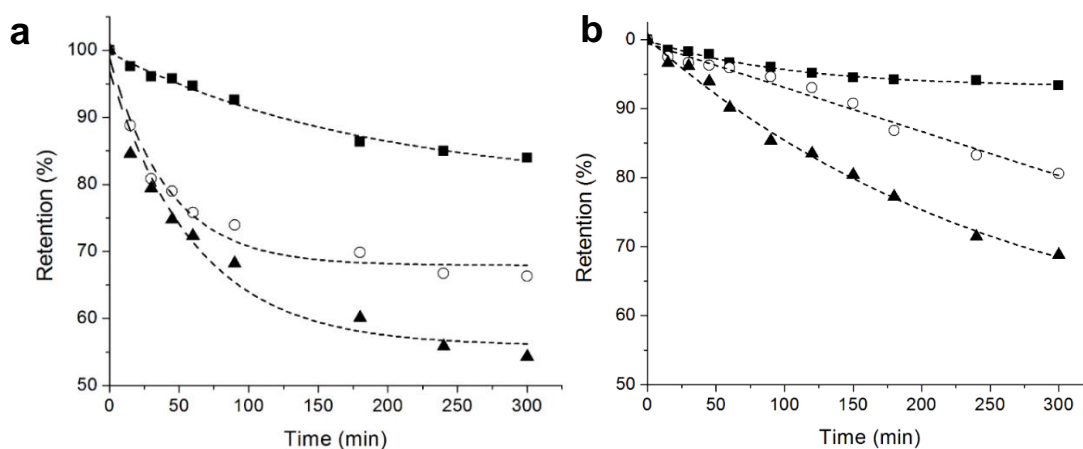


Fig.3 Norbixin retention (%) during heating experiment a) Free norbixin (F-Norb), b) Microcapsules (MIC-Norb) with temperature 60°C (■), 90°C (○), and 98°C (▲), respectively

The k values was used to calculate the half-life time ($t_{1/2}$) for each temperature, this parameter indicates the time required for norbixin to reduce a 50% of its initial measurement. As it can be observed, $t_{1/2}$ depends of temperature and for all experiments $t_{1/2}$ was higher to MIC-Norb (Table 1). Increase in temperature induced a smaller $t_{1/2}$ due to use of accelerated treatments and inducing a faster degradation of compounds. When was evaluated the thermal stability with the lower temperature (60°C) the result of $t_{1/2}$ for MIC-Norb was approximately 4.5 times greater than $t_{1/2}$ value for F-Norb. This means that encapsulated norbixin showed a higher half-life time than free norbixin and is possible to predict that MIC-Norb are able to maintain their quality for long time if be used food storage temperatures (4 – 25°C). Thus, confirming that encapsulation is an alternative to enhance a compound's shelf life. The activation energy (E_a) for F-Norb and MIC-Norb was determined using the Arrhenius equation; results are shown in Table 1. The value of E_a is a measure of the sensitivity of the reaction to temperature, i.e., when E_a is lower, means that compound has more sensitivity to degradation, then loss from F-Norb occurs first than from MIC-Norb, showing the encapsulated compound has upper resistance to high temperatures. MIC-Norb needs approximately double of E_a (15.08 kcal/mol) for thermal degradation than F-Norb (7.61 kcal/mol). Thus, in this study was demonstrated that norbixin is more stable when is encapsulated than it is exposed to heating in free form. For all experiments, the losses were higher for F-Norb, showing the microencapsulation techniques offer greater protection to norbixin. The E_a value

obtained for thermal degradation of MIC-Norb in this research could be used to predict the kinetic behavior at higher temperatures. All kinetic degradation reactions were of first order, ratifying this model best describes the behavior of norbixin during thermal degradation (Silva et al. 2007).

Table 1 Rate degradation constant (k), activation energy (Ea) and half-life time for free norbixin (F-Norb) and norbixin microcapsules (MIC-Norb).

Sample	F-Norb			MIC-Norb		
	60°C	90°C	98°C	60°C	90°C	98°C
Rate constants						
$k_t \times 10^{-4} \text{ (min}^{-1}\text{)}$	5.90	16.23	18.54	1.30	8.10	13.78
$t_{1/2} \text{ (h)}$	19.59	7.12	6.23	88.82	14.26	8.39
$E_a \text{ (kcal/mol)}$		7.61			15.08	

Parvin et al. (2011) reported the same effect in the study on thermal stability of free norbixin extracts and obtained a similar E_a value of 5.15 kcal/mol. As expected, with temperature and time increased, norbixin concentration decreased, a similar tendency to reported in stability studies of astaxanthin (Bustos-Garza et al. 2013) and curcumin microcapsules (Wang et al. 2009) prepared by spray-drying.

The use of GA as a wall material to carry out microcapsules by spray-drying increases $t_{1/2}$ and significantly influences the increase in shelf life of bioactive compounds when was compared to other polymers (Kanakdande et al. 2007). A good retention and protection of compounds in GA is due to good emulsifying properties and ability of this carbohydrate to form film around of constituent of interest, thus offering a plasticity property that prevents the cracking of the protection matrix (Krishnan et al. 2005).

According to Castellani et al. (2010), the emulsifying characteristics in water and good capability film forming of GA is due to the properties of arabinogalactan protein (AGP), molecules presents in this polymer.

On the other hand, Guan and Zhong (2014) reported that norbixin is able to form complexes with GA, but the bonds that were formed were unstable because only dispersions were used as a encapsulation matrix. Possibly, the drying process in this study strengthened those complexes and allowed core norbixin to become more thermo resistant.

The thermal degradation kinetic study for commercial norbixin solutions, performed by Ferreira et al. (1999), showed the same effect and the authors concluded that color alterations and norbixin content decrease depend on temperature and time of treatment. Color degradation reactions and norbixin concentration losses were less significant at temperatures lower 100°C than at higher temperatures (140°C). Over 100°C other colored compounds are produced through isomeric reactions, like m-xylene, toluene and dihydronaftalen, which damage human health, so storage stability of norbixin is highly influenced by the ability of the compound to exhibit a specific color (Gallardo-Cabrera and Rojas-Barahona 2015).

Barbosa et al. (2005) found the same first order kinetic behavior in the thermal stability study of spray-drying encapsulated bixin with GA. Both free and encapsulated bixin were exposed to the same temperatures and both showed the same effect. Scotter (2009) and Silva et al. (2007) reported that thermal degradation of bixin and norbixin followed the first order kinetic effect in their study. Therefore the rapid decrease in F-Norb and MIC-Norb retention (Fig. 3) at the beginning of the heating test is due to the non-encapsulated material that remained on surface of capsules and was consequently exposed directly to high temperatures (Barbosa et al. 2005; Lobato et al. 2015; Tonon et al. 2010).

Most stability studies for bioactive compounds use accelerated treatments with extreme temperature conditions to ease the shelf life estimation, however, Prabhakara Rao et al. (2002) evaluated thermal stability of free norbixin and norbixin in an aqueous solution (fresh orange juice) using refrigeration temperature (7 - 10°C) and room temperature (25 - 30°C) to simulate storage conditions for a long period (5 months). As a result, the authors reported significant loss in norbixin concentration and color attributes for experiments at room temperature, but at low temperatures, the degradation losses were not significant.

Other studies on thermal stability of spray-drying microencapsulated bioactive compounds proved a higher compound loss by degradation when exposed to high temperatures in free form than when exposed as microcapsules; such is the case of lycopene (46%) (Rocha et al. 2012), and also reported a first order kinetic behavior for betalains (Saéñz et al. 2009) and astaxanthin (Bustos-Garza et al. 2013).

4 Conclusion

In the present study, it was possible to determine the kinetics of thermal degradation of norbixin microcapsules. Activation energy required for degradation was very higher (double) than the energy required for free norbixin when both were compared using the same model system. Therefore, it was proven that spray-drying microencapsulation using gum arabic as wall material is an excellent alternative to enhance the thermal stability of norbixin and thus offer a longer shelf life. This turns norbixin microcapsules into an attractive food additive to be incorporated into food matrices, especially into beverages due to their hydro-solubility.

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SUPPLEMENTARY DATA

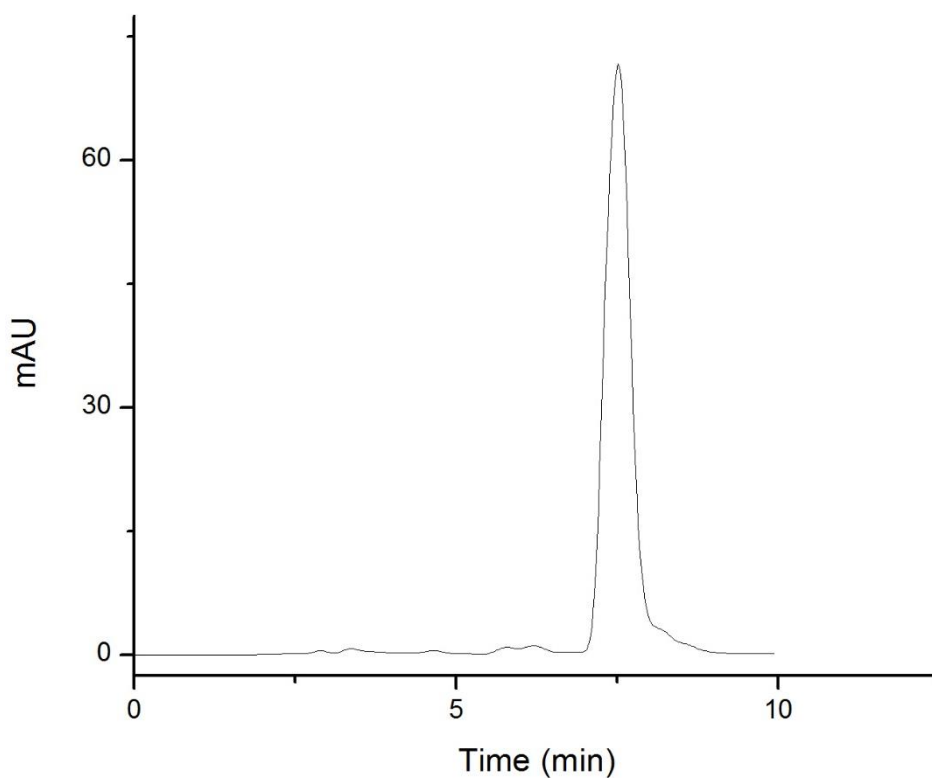


Fig. A1. HPLC chromatogram of norbixin crystals

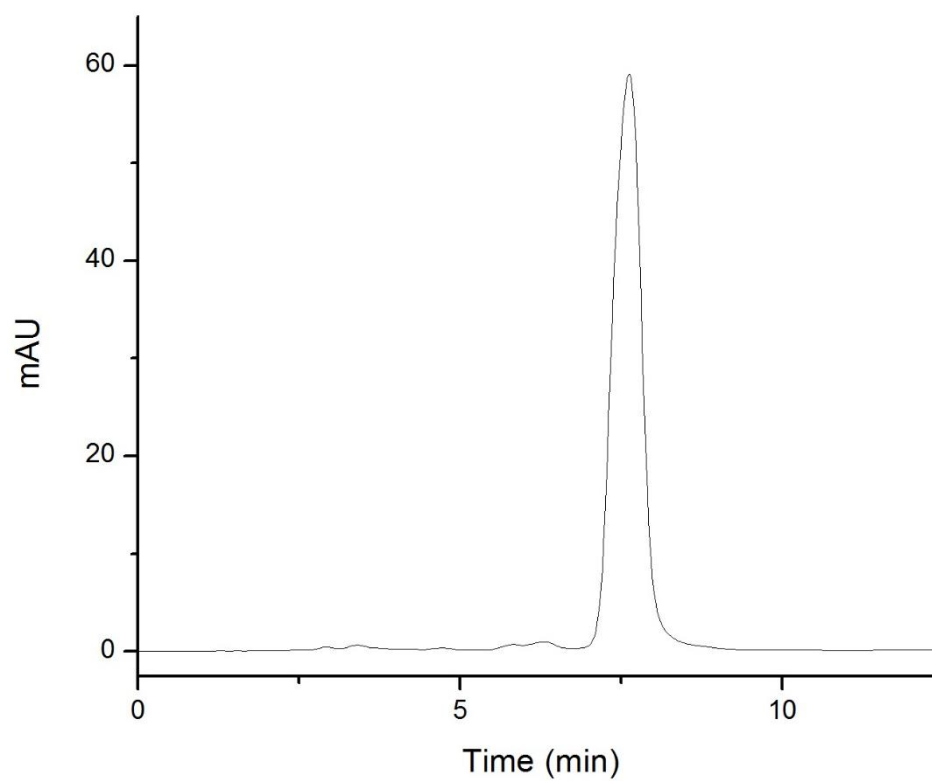


Fig. A2. HPLC chromatogram of norbixin microcapsules

ARTIGO 3

**STABILITY ASSESSMENT OF ISOTONIC TANGERINE SOFT DRINK ADDED
OF NORBIXIN MICROCAPSULES OBTAINED BY SPRAY DRYING AS A
NATURAL DYE**

Artigo formatado segundo as normas da revista "Food Chemistry"

Stability assessment of isotonic tangerine soft drink added of norbixin microcapsules obtained by spray drying as a natural dye

Diego Santiago Tupuna, Karina Paese, Silvia Stanisçuaski Guterres, Simone Hickmann Flôres, Alessandro de Oliveira Rios

ABSTRACT

Annatto seeds (*Bixa orellana* L.) are a natural source of norbixin, a carotenoid with antioxidant activity and intense yellow-orange color, commonly used to provide coloration to foods and beverages. Nevertheless, it is susceptible to environmental factors such as light, oxygen, and temperature. Microencapsulation is an alternative for improving the stability of bioactive compounds. In this study were applied norbixin microcapsules (MCN) in isotonic tangerine soft drinks without exceeding the food additives regulations. The final concentration was $2.86 \pm 0.02 \mu\text{g}$ norbixin/mL and according to CIELab system, the beverage got an orange tonality as was expected. The addition of MCN affects the stability of beverages favorably throughout storage under accelerated conditions (heat and light), the half-life time was greater (29.71 days) than when used norbixin non-encapsulated (6.56 hours). In conclusion, the MCN are a natural dye with potential use in processed beverage industries as a substitute for synthetic dyes.

1 Introduction

Color is one of the main attributes in processed food that influences their preference and acceptance directly, moreover, it is considered a quality indicator and a visual aspect frequently related to taste identification. (Rios & Mercadante, 2004). In recent years, the increase of the consumer concern and food legislation about negative effects of synthetic dyes in human health promote the interest in the development of natural dyes from natural sources (Mesnier, Gregory, Fañca-Berthon, Boukobza, & Bily, 2014; Özkan & Bilek, 2014).

Norbixin is an apocarotenoid (9'-*cis*-6,6'-diapocarotene-6,6'-dioic acid) *cis*-norbixin water-soluble, their solubility and stability due to its chemical structure (Scotter, 2009). The annatto extracts have a lower cost in relation to other natural pigments and are used as a natural colorant in different food matrix (butter, cheese, bakery products, ice cream, sausages, extruded products, salad dressings, cereals, sodas and liquors (Cardarelli, Benassi, & Mercadante, 2008).

Norbixin can confer an intense coloration in the orange-yellow-red range according to the final concentration of the compound (Satyanarayana, Rao, & Rao, 2010; Scotter, 2009). It is used as a food additive since it has no adverse toxicological effects on humans when ingested in minimal quantities on a daily intake (Hagiwara et al. 2003). In order to achieve a typical fruit coloration in non-alcoholic beverages are highly used synthetic dyes Sunset Yellow (E110), Tartrazine (E102) and Amaranth (E123), which contain azo (N=N) functional groups and aromatic ring structures that are harmful to human health. The high consumption of products with synthetic colorants is related to diseases linked to allergies in susceptible people (De Andrade et al., 2014). Therefore, The Brazilian National Agency for Public Health Surveillance (ANVISA) established legal regulations about the use of synthetic food dyes and their allowed levels in all food products (ANVISA, 2006). The use of norbixin would be an alternative to replace the use of synthetic dyes in isotonic soft drinks.

In addition to its ability to provide color, norbixin is an amphipathic molecule with antioxidant activity due to its multiple conjugated double bonds (Guan & Zhong, 2014). Nonetheless, this chemical structure induce a susceptibility to degradation when exposed to adverse factors (high temperatures, oxygen, light, low pH), which reduces its stability and limit its application, shelf life and bioavailability in food products because are subjected to quality loss during storage (DJordjević et al., 2015; Kiokias & Gordon, 2003; Montenegro, Rios, Mercadante, Nazareno, & Borsarelli, 2004; Rodriguez-Amaya, Rodriguez, & Amaya-Farfan, 2006).

With the purpose of increase the stability of carotenoids, techniques of encapsulation are considered one of the most important alternatives. Microencapsulation is defined as a drying process in which solid micro particles, gas compounds or liquid droplets are surrounded by a coating material that forms a polymeric film around the core, acting as a protection barrier (Gharsallaoui, Roudaut, Chambin, Voilley, & Saurel, 2007). Many encapsulation processes have been developed. Nevertheless, a spray-drying method is the most commonly used in food industry due to its efficiency and low cost (Kandansamy & Somasundaram, 2012).

Gum Arabic is a wall material most frequently used in encapsulation process due to its emulsifying ability, film-forming capacity and low viscosity at high temperatures. This carbohydrate shows a high encapsulation efficiency and antioxidant activity when was compared with other coating materials used to encapsulate bioactive compounds (Barbosa, Borsarelli, & Mercadante, 2005; Cano-Higueta, Malacrida, & Telis, 2015;

Ravichandran et al., 2014; Rodrigues, Mariutti, Faria, & Mercadante, 2012; Stoll, Costa, Jablonski, Flôres, & de Oliveira Rios, 2016).

The aim of this study was to assess the stability of isotonic tangerine soft drink added of norbixin microcapsules as natural dye during storage to analyze the kinetics of degradation when beverages were exposed for a long time to accelerated conditions of light and heat. In order to define an estimated shelf life for future applications of norbixin as a natural bioactive dye in isotonic beverages.

2 Materials and methods

2.1 Materials

The annatto seeds were acquired from the local market in Porto Alegre, RS, Brazil. As a carrier agent of encapsulation was used an edible polysaccharide Gum Arabic SYNTH (Porto Alegre, RS, Brazil). All reactants were of analytical grade.

2.2 Preparation of the norbixin crystals

To obtain a purified norbixin extract was followed the procedure described by Rios & Mercadante (2004). First, the annatto seeds were washed with organic solvents in order to achieve remove impurities and were performed an extraction of bixin with ethyl acetate; the extract was dried in a rotary evaporator Fisatom M802 (São Paulo, Brazil). Then, was used dichloromethane to dissolve the extract and immediately absolute ethanol was added for the formation of bixin crystals. Finally, was carried out a saponification of bixin crystals using a methanolic KOH solution (10%), the obtained norbixin salt was separated through a separation funnel, and the obtained norbixin crystals (NC) was dried in a rotary evaporator and stored at -18°C.

2.3 Quantification of norbixin

A concentration of norbixin was quantified by spectrophotometry using a UV Spectrophotometer UV-1800 Shimadzu (Tokyo, Japan) at 453 nm (Alves, De Souza, & Jauregi, 2006) and absorptivity value $E_{1\text{cm}}^{1\%} = 3473$ (Yabiku, & Takahashi, 1992). First, norbixin crystals were dissolved in ethanol (99.7%) and an aliquot was diluted in a KOH (0.5%) aqueous solution that was used as a standard. To measure a norbixin in isotonic beverages was not necessary to perform the first dissolution in ethanol, directly an

aliquot of each sample of beverage was taken and diluted in KOH (0.5%). A standard curve was developed for the quantification of norbixin with a correlation factor (R^2) de 0.99. For each calculation, was considered along with the following equation:

$$x_{(\mu\text{g})} = \text{Abs} \times \gamma \times 10^6 / E \times 100 \quad (1)$$

Abs = Absorbance

γ = dilution (mL KOH 0.5%)

E = Absorptivity coefficient

2.4 *Microencapsulation by Spray-drying*

A formulation was prepared by dissolution of Gum Arabic (GA) in 80 mL of distilled water until achieving 40% total soluble solids (TSS) and added 30 mg NC diluted in 20 mL ethanol (99.7%). The solution was magnetically stirred at 40°C for 30 min until reach a homogeneous emulsion that was stored at -4°C.

The drying process was carried out in a Mini Spray Dryer Büchi B-290 (Flawil, Switzerland) to get a microencapsulation. The feed flow 6.6 mL/min and 150°C inlet temperature were used as operation parameters in equipment and was performed experiments in duplicate. Immediately the microcapsules were vacuum packed in plastic bags and stored at -4°C.

As a result of characterization of norbixin microcapsules (MCN) these showed a low moisture value of $3.00 \pm 0.50\%$, particle size distribution monomodal with a span value of 1.96 ± 0.25 and volume-weighted mean diameter $D_{(4.3)}$ of $8.25 \pm 0.83 \mu\text{m}$. Additionally was carried out an ABTS test and MCNs exhibited antioxidant activity ($87.65 \pm 0.46 \mu\text{mol TE/g dry sample}$). MCNs had a high water solubility index ($92.04 \pm 0.75\%$) and high microencapsulation efficiency ($70.97 \pm 1.48\% - 386.25 \pm 0.18 \mu\text{g/g microcapsules}$) as consequence of successful encapsulation process.

2.5 *Preparation of simulated isotonic tangerine soft drink (ITSD)*

Colorimetry of commercial isotonic tangerine soft drink (ITSD) was analyzed with a colorimeter Chromameter CR-400 Konica Minolta(Tokyo, Japan) with the aim to define the quantity of norbixin to be added in ITSD in order to get a beverage with similar values of CIELab color parameters ($L^* = 48.27 \pm 0.01$, $a^* = 6.75 \pm 0.04$, $b^* = 22.76 \pm 0.01$). At

the same time without exceeding the limit established to norbixin E160b (0.005 g/100 mL) as additive in non-alcoholic beverages by Brazilian food legislation (ANVISA, 2006). After several previous experiments, the norbixin concentration that achieved the desired color tonality (orange) according to CIELab color chart was in the range from 2.50 to 2.90 $\mu\text{g/mL}$. Hence, we have aimed a beverage with norbixin concentration in this range. Then two ITSD working stock solutions were prepared, each one with 1250 mL of distilled water containing 50 g of crystalline sugar, 875 mg of KCl, 1125 mg of NaCl, 375 mg of citric acid, 375 mg of ascorbic acid and 375 μL of artificial tangerine flavor. In one of these was dissolved 10 g of MCN until achieve a beverage with final concentration of $2.86 \pm 0.02 \mu\text{g/mL}$ of norbixin with the aim to get an ITSD colored with MCN (Isotonic-MCN), and for control, in the other was added 650 μg of NC until achieve a beverage with final concentration of $2.56 \pm 0.05 \mu\text{g/mL}$ of norbixin, this was an ITSD colored with NC (Isotonic-NC). All solutions were stored at -4°C in the dark.

2.6 Storage stability studies

2.6.1 Acidity, pH, total soluble solids ($^\circ\text{Brix}$)

Immediately after preparation, the beverages Isotonic-MCN and Isotonic-NC were chemically characterized to obtain the initial value of acidity, pH, $^\circ\text{Brix}$. During storage was periodically taken a sample of each beverage in time intervals and were also analyzed. The acidity was determined by standard procedures, the samples were diluted with distilled water, then a few drops of ethanolic phenolphthalein (1%) were added, and the mixture was titrated with aqueous NaOH 0.01 N until color change and was expressed as mg citric acid/L of sample. The TSS was measured by using a refractometer Pocket ATAGO model PAL-3 (Tokyo, Japan) and was expressed in $^\circ\text{Brix}$. To evaluate pH was used a pH meter QUIMIS (São Paulo, Brazil).

2.6.2 Colorimetric Analysis

The color change of the beverages was monitored using a colorimeter Chromameter CR-400 Konica Minolta (Tokyo, Japan). Measurements was recorded according to the CIELAB system with the parameters L^* , a^* , b^* , where L^* is the lightness (0 = white) to darkness (100 = black) variation, a^* the variation between green ($<a^*$) and red ($>a^*$), and b^* the variation between blue ($<b^*$) and yellow ($>b^*$). A white ceramic disc was applied for calibration before carrying out any measurements. These values were then used to calculate the Chroma value (C^*), which indicate vividness of color

saturation, Hue angle system (h°_{ab}) that implies the color tone of the sample (0 or 360 = red, 90 = yellow, 180 = green and 270 = blue) and the total color difference (ΔE) throughout storage as follows (Kim, Park, & Hwang, 2002):

$$C^* = (a^*)^2 + (b^*)^2 \quad (2)$$

$$(\Delta E) = [(L_o^* - L^*)^2 + (a_o^* - a^*)^2 + (b_o^* - b^*)^2]^{1/2} \quad (3)$$

$$h^{\circ}_{ab} = \tan^{-1} (b^*/a^*) \quad (4)$$

2.7 Stability of beverages on accelerated conditions of storage

Trials were conducted to assess the loss of color during accelerated conditions of storage in order to establish temperature and period of experimental assays. The beverages were distributed in transparent plastic bottles with screw caps, 30 mL in each one and immediately were flushed with nitrogen, closed and sealed with a plastic paraffin film. To assess the influence of the wall material on light stability of norbixin, two sets of experiments were carried out at a specific temperature in a laboratory oven. For Isotonic-NC the samples were stored under fluorescent light (ca. 750 lx) or in the dark at $35 \pm 2^{\circ}\text{C}$ and the norbixin concentration, color, $^{\circ}\text{Brix}$, acidity and pH in samples were measured at 0, 30, 60, 180, 240, 300, 420 and 500 min during storage. For Isotonic-MCN the samples were stored under fluorescent light (ca. 750 lx) or in the dark at $40 \pm 2^{\circ}\text{C}$ and the same parameters in samples were measured at 0, 1, 3, 5, 7, 10, 12, 15 days during storage. All experiments were performed in triplicate.

The degradation kinetics of norbixin were analyzed using the first-order reaction as has previously been reported by (Silva, Souza, Botelho, Silva, & Silva, 2007; Scotter, 2009). To calculate the kinetic parameters the norbixin retention (%) was plotted against time. The degradation rate constant (k_t) was determined from the first derivative of the curves plotted with software Origin 8.0 (Origin Lab Co., MA, USA) (de Rosso & Mercadante, 2007). Half-life time ($t_{1/2}$) was calculated following the equation (Bustos-Garza, Yáñez-Fernández, & Barragán-Huerta, 2013):

$$[\text{norbixin}] = [\text{norbixin}]_0 \times \exp (-k_t \times t) \quad (5)$$

$$t_{1/2} = \ln 2 / k_t \quad (6)$$

Where: $t_{1/2}$ = Half-life time value; k_t = Kinetic constant; t = time

2.8 *Statistical Analysis*

The statistical parameters were calculated by using STATGRAPHICS Centurion software. One-way analyses of variance (ANOVA) and a multiple-range test were performed to correlate the data and to establish statistically significant differences ($p < 0.05$) with a 95% confidence interval. All results were recorded and expressed as mean value \pm standard deviation.

3 **Results and Discussion**

3.1 *Chemical characterization of beverages during storage*

The measurements of chemical parameters at time zero in working solutions of beverages were considered as the initial value of storage studies in order to compare with the final values to determine significant variations over time. The beverages were analyzed for acidity, pH and TSS and results are presented in Table 1.

During storage, these parameters in each beverage showed different behavior in presence or absence of light (Table 1). TSS and acidity were highest for Isotonic-MCN due to the presence of GA. In both solutions of beverages with NC and MCN as natural dye a statistically significant variation ($p < 0.05$) between initial and final value can be observed in all parameters throughout the storage time in accelerated conditions of temperature and exposed to light. However, when the final value was compared with the final value in the dark, the influence of light was not shown significant differences ($p > 0.05$) (Table 1). For pH there was a minimum decrease in all experiments, it means that norbixin can be stable in acidic beverages during storage in agreement with Prabhakara Rao, Satyanarayana, & Rao (2002) and Guan & Zhong (2014) who reported the same tendency when was added in orange juice and acidified aqueous norbixin solutions, respectively.

When Bustos-Garza et al. (2013) evaluated the pH stability of astaxanthin encapsulated with different wall materials and reported the high $t_{1/2}$ at pH values 3.0 and 4.0 when was used only GA as a coating material. Additionally, when astaxanthin was added as a pigment to acidic food matrix and model instant drinks showed stable behavior (Anarjan & Tan, 2013; Villalobos-Castillejos, Cerezal-Mezquita, Hernández-De Jesús, & Barragán-Huerta, 2013).

3.2 *Stability of beverages on accelerated conditions of storage*

3.2.1 *Color changes during storage*

The data of colorimetry analyses of Isotonic-NC and Isotonic-MCN samples at time zero were located in the first quadrant of the CIELab color chart. The beverages showed values from 5.73 to 6.15 and 20.48 to 24.58 for a^* and b^* , respectively (Fig. 1). The h°_{ab} values ranged from 74.36 to 75.96 (Table 2.) and indicated that was possible to reach beverages with a typical orange coloration of isotonic tangerine soft drink by using norbixin as a natural dye. Prabhakara Rao, Satyanarayana, & Rao (2002) reported the same tendency when norbixin was applied in orange juice ready-to-serve and achieved the desired orange yellow color at very low concentrations.

In contrast, when was evaluated astaxanthin stability incorporated as a natural food colorant in a model instant drink, Villalobos-Castillejos, Cerezal-Mezquita, Hernández-De Jesús, & Barragán-Huerta (2013) reported that this carotenoid showed change from intense orange color to pink when it was dispersed in water. This behavior confirms that norbixin did not present changes in its coloration when it is dissolved in aqueous solution, as is the case with other carotenoids as was the case with other carotenoids.

The color stability of norbixin in Isotonic-NC stored at 35°C, under light or dark, was determined. For both conditions were observed significant differences ($p < 0.05$) between initial and final values of CIELab parameters, occurs an increase of L^* and a^* , b^* showed an extensive color fading (Fig. 1a). Nevertheless, in the absence of light, the norbixin color was more stable throughout storage. An increase of parameter L^* means a loss of color that causes a lightness tonality of beverage, while a decrease of parameter a^* and b^* indicates a change of color from orange to yellow, because of redness loss. For experiments with Isotonic-NC samples the parameters L^* and a^* showed the greater change after 60 minutes of storage, and endure stable more time (Fig. 1a). In the presence of light, the color changes were more pronounced above all for parameter b^* ; this means that the light was more responsible for the color variation of Isotonic-NC than the influence of temperature. Many other authors also reported that the higher decrease of parameter b^* happens during degradation of carotenoids represents the most interest to stability studies (Lim, Burdikova, Sheehan, & Roos, 2016; Mesnier et al., 2014), because it means a loss of yellowness that causes a change in desired shade of beverage.

Table 1. Measurements of acidity, pH and TSS for isotonic tangerine soft drinks added of non-encapsulated norbixin and norbixin microcapsules during storage under accelerated conditions of light and temperature.

Isotonic-NC						
Time (min)	pH		Total soluble solids (°Brix)		Acidity (mg citric acid/L)	
	Dark	Light	Dark	Light	Dark	Light
0	3.21 ± 0.01 ^{aA}	3.21 ± 0.01 ^{aA}	3.60 ± 0.01 ^{aA}	3.60 ± 0.01 ^{aA}	0.12 ± 0.01 ^{aA}	0.12 ± 0.01 ^{aA}
30	3.20 ± 0.01 ^{aA}	3.19 ± 0.01 ^{bB}	3.63 ± 0.06 ^{aA}	3.83 ± 0.06 ^{cdB}	0.14 ± 0.01 ^{abA}	0.16 ± 0.02 ^{bA}
60	3.18 ± 0.01 ^{bA}	3.17 ± 0.01 ^{cA}	3.77 ± 0.06 ^{bcA}	3.70 ± 0.03 ^{abB}	0.15 ± 0.01 ^{bA}	0.17 ± 0.02 ^{bcA}
180	3.10 ± 0.02 ^{efA}	3.11 ± 0.01 ^{dA}	3.73 ± 0.06 ^{bA}	3.73 ± 0.15 ^{bcA}	0.15 ± 0.01 ^{bA}	0.17 ± 0.02 ^{bcA}
240	3.13 ± 0.01 ^{cA}	3.11 ± 0.01 ^{dB}	3.83 ± 0.06 ^{cdA}	3.97 ± 0.06 ^{eB}	0.16 ± 0.02 ^{bA}	0.17 ± 0.03 ^{bcA}
300	3.12 ± 0.01 ^{cdA}	3.11 ± 0.01 ^{dA}	3.90 ± 0.02 ^{dA}	3.90 ± 0.02 ^{deA}	0.16 ± 0.01 ^{bA}	0.18 ± 0.01 ^{bcB}
420	3.11 ± 0.02 ^{deA}	3.10 ± 0.01 ^{dA}	4.00 ± 0.02 ^{dA}	3.90 ± 0.01 ^{deA}	0.17 ± 0.03 ^{bcA}	0.18 ± 0.02 ^{bcA}
500	3.09 ± 0.02 ^{fA}	3.09 ± 0.01 ^{eA}	3.90 ± 0.01 ^{dA}	3.90 ± 0.03 ^{deA}	0.19 ± 0.02 ^{cA}	0.19 ± 0.03 ^{cA}

Isotonic-MCN						
Time (days)	pH		Total soluble solids (°Brix)		Acidity (mg citric acid/L)	
	Dark	Light	Dark	Light	Dark	Light
0	3.89 ± 0.01 ^{aA}	3.89 ± 0.01 ^{aA}	5.17 ± 0.06 ^{aA}	5.17 ± 0.06 ^{aA}	0.20 ± 0.03 ^{aA}	0.20 ± 0.03 ^{abA}
1	3.89 ± 0.01 ^{aA}	3.85 ± 0.03 ^{bB}	5.73 ± 0.12 ^{aA}	5.77 ± 0.12 ^{dA}	0.21 ± 0.02 ^{abA}	0.20 ± 0.02 ^{aA}
3	3.82 ± 0.01 ^{bA}	3.77 ± 0.01 ^{cB}	5.23 ± 0.06 ^{abA}	5.37 ± 0.06 ^{bA}	0.22 ± 0.01 ^{abcA}	0.22 ± 0.03 ^{abcA}
5	3.83 ± 0.01 ^{bA}	3.83 ± 0.03 ^{bA}	5.57 ± 0.06 ^{deA}	5.50 ± 0.04 ^{cA}	0.24 ± 0.03 ^{bcA}	0.24 ± 0.02 ^{bcA}
7	3.74 ± 0.02 ^{dA}	3.75 ± 0.02 ^{deA}	5.37 ± 0.06 ^{bcA}	5.40 ± 0.13 ^{bB}	0.24 ± 0.04 ^{bcA}	0.24 ± 0.01 ^{cB}
9	3.78 ± 0.01 ^{cA}	3.76 ± 0.01 ^{cdA}	5.50 ± 0.26 ^{cdA}	5.10 ± 0.08 ^{aB}	0.24 ± 0.01 ^{dA}	0.24 ± 0.02 ^{cA}
12	3.75 ± 0.02 ^{dA}	3.74 ± 0.01 ^{deA}	5.33 ± 0.06 ^{abcA}	5.43 ± 0.06 ^{bA}	0.24 ± 0.03 ^{dA}	0.24 ± 0.05 ^{bcB}
15	3.74 ± 0.02 ^{dA}	3.73 ± 0.02 ^{eA}	5.63 ± 0.06 ^{bdeA}	5.70 ± 0.01 ^{dA}	0.24 ± 0.02 ^{dA}	0.24 ± 0.03 ^{cA}

Mean ± standard deviation (n=3); Different lowercase letters in the same column indicate statistically significant differences between samples at p<0.05 and different capital letters in the same file indicate statistically significant differences between dark or light measurements for each parameter at p<0.05 (Isotonic-NC: Isotonic tangerine soft drink with norbixin crystals, Isotonic-MCN: Isotonic tangerine soft drink with norbixin microcapsules)

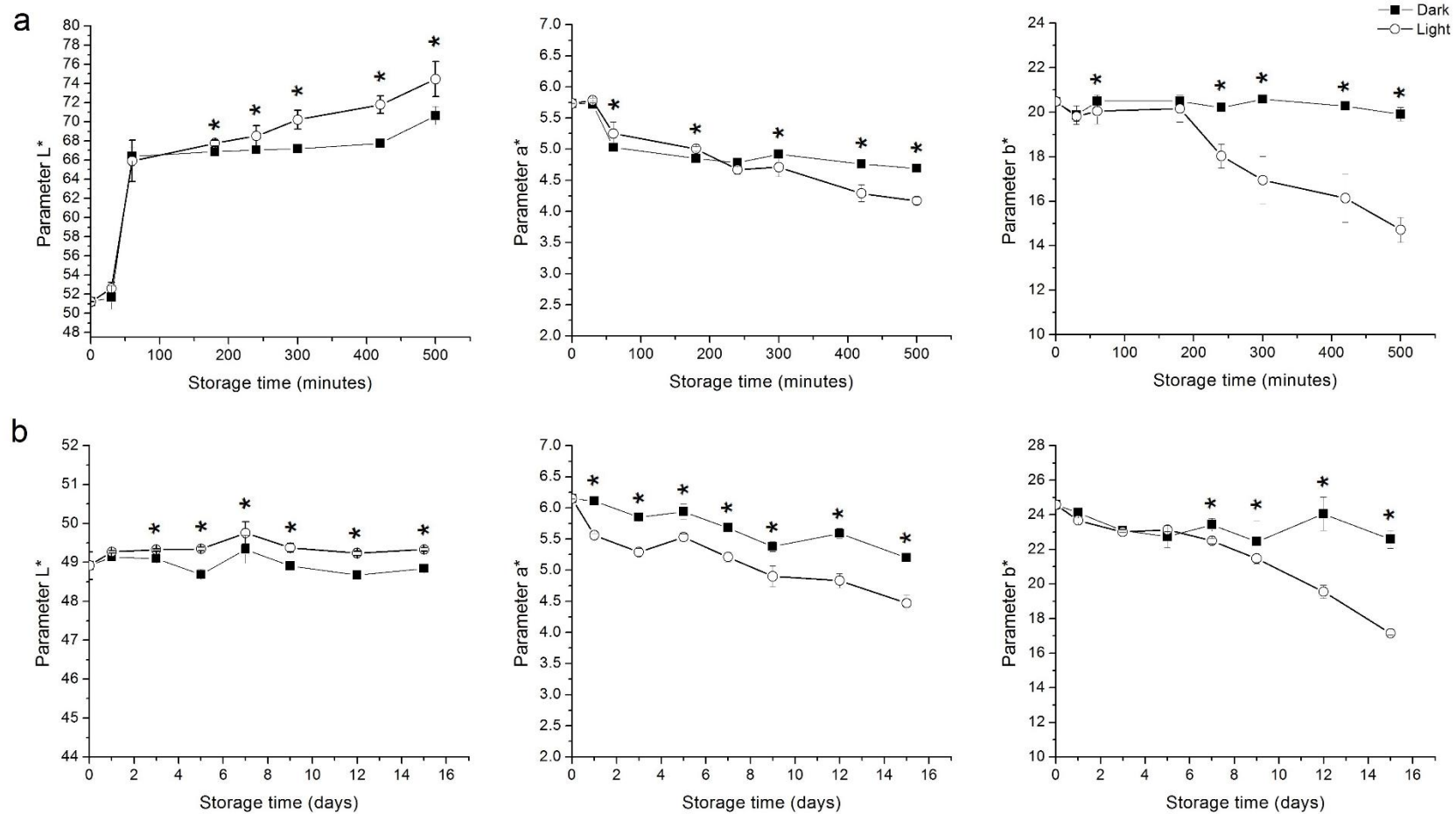


Fig. 1. Changes of L^* (Lightness), a^* (redness) and b^* (yellowness) color parameters: a) Isotonic tangerine soft drinks samples prepared with norbixin crystals (Isotonic-NC) during storage at 35°C, b) Isotonic tangerine soft drinks samples prepared with norbixin microcapsules (Isotonic-MCN) during storage at 40°C. (*) Statistically significant differences ($p < 0.05$) between dark and light results.

Table 2. Color parameters Chroma, Hue, ΔE for isotonic tangerine soft drinks added of non-encapsulated norbixin and norbixin microcapsules during storage under accelerated conditions of light and temperature.

Isotonic-NC						
Sample	Chroma		Hue		ΔE	
Rate parameters	Dark	Light	Dark	Light	Dark	Light
Time (min)	Dark	Light	Dark	Light	Dark	Light
0	21.27 ± 0.01 ^a	21.27 ± 0.01 ^a	74.36 ± 0.01 ^b	74.36 ± 0.01 ^{abc}	0.00	0.00
30	20.68 ± 0.38 ^{cd}	20.63 ± 0.19 ^a	73.93 ± 0.46 ^a	73.72 ± 0.27 ^a	1.29 ± 0.15	1.60 ± 0.51
60	21.11 ± 0.25 ^{ab}	20.73 ± 0.57 ^a	76.22 ± 0.32 ^c	75.32 ± 0.58 ^{bcd}	15.19 ± 1.00	14.73 ± 2.18
180	21.06 ± 0.05 ^{ab}	20.77 ± 0.57 ^a	76.69 ± 0.08 ^d	76.05 ± 0.51 ^d	15.70 ± 0.39	16.58 ± 0.30
240	20.77 ± 0.08 ^{cd}	18.62 ± 0.54 ^b	76.69 ± 0.19 ^d	75.47 ± 0.19 ^{cd}	15.90 ± 0.41	17.52 ± 1.00
300	21.17 ± 0.04 ^a	17.60 ± 0.99 ^{bc}	76.56 ± 0.20 ^{cd}	74.42 ± 1.41 ^{abcd}	15.99 ± 0.11	19.39 ± 0.79
420	20.82 ± 0.08 ^{bc}	16.69 ± 1.06 ^c	76.79 ± 0.09 ^d	75.07 ± 0.93 ^{bc}	16.59 ± 0.17	21.10 ± 0.78
500	20.46 ± 0.29 ^d	15.29 ± 0.54 ^d	76.74 ± 0.25 ^d	74.16 ± 0.65 ^{ab}	19.47 ± 0.93	24.01 ± 1.81

Isotonic-MCN						
Sample	Chroma		Hue		ΔE	
Rate parameters	Dark	Light	Dark	Light	Dark	Light
Time (days)	Dark	Light	Dark	Light	Dark	Light
0	25.34 ± 0.26 ^a	25.34 ± 0.26 ^a	75.96 ± 0.16 ^c	75.96 ± 0.16 ^b	0.00	0.00
1	24.88 ± 0.06 ^{ab}	24.32 ± 0.14 ^b	75.78 ± 0.13 ^{de}	76.79 ± 0.08 ^{de}	0.62 ± 0.18	1.19 ± 0.25
3	23.81 ± 0.09 ^{cd}	23.60 ± 0.12 ^c	75.78 ± 0.07 ^{de}	77.05 ± 0.19 ^e	1.57 ± 0.25	1.87 ± 0.12
5	23.50 ± 0.65 ^{cd}	23.78 ± 0.11 ^c	75.36 ± 0.23 ^{de}	76.55 ± 0.07 ^{cd}	1.88 ± 0.97	1.68 ± 0.28
7	24.01 ± 0.36 ^{bc}	23.09 ± 0.13 ^c	76.37 ± 0.21 ^c	76.96 ± 0.27 ^{de}	1.40 ± 0.52	2.51 ± 0.09
9	23.09 ± 1.16 ^d	22.02 ± 0.27 ^d	76.50 ± 0.59 ^b	77.14 ± 0.54 ^e	2.31 ± 1.39	3.40 ± 0.35
12	24.04 ± 0.41 ^{bc}	20.14 ± 0.39 ^e	76.55 ± 0.06 ^b	76.12 ± 0.13 ^{bc}	1.38 ± 0.25	5.22 ± 0.46
15	23.17 ± 0.51 ^{cd}	17.73 ± 0.11 ^f	77.03 ± 0.27 ^a	75.40 ± 0.39 ^a	2.25 ± 0.51	7.63 ± 0.29

Mean ± standard deviation (n=3)

Different letters in the same column indicate statistically significant differences between samples at p<0.05

(Isotonic-NC: Isotonic tangerine soft drink with norbixin crystals, Isotonic-MCN: Isotonic tangerine soft drink with norbixin microcapsules)

The results obtained for Isotonic-MCN samples stored at 40°C, under light or in the dark showed that norbixin encapsulated could be more stable during storage (Fig. 1b.), in absence of light although existed significant differences (p<0.05) for parameters a^* and b^* , these presented a little variation (Fig. 1b). Parameter L^* did not show significant changes, this may be due to the presence of GA in MCNs. Its known that this polysaccharide is commonly used as an emulsifier and thickening agent in soft drinks with the aim to produce cloudy products and improves the shelf life (Ashurst, 2016). L^* values presented the same behavior when was evaluated the addition on GA in acidified

aqueous solutions of norbixin to improve their thermal and acid stabilities (Guan & Zhong, 2014).

In the presence of light was observed a high decrease of parameters a^* and b^* during a storage time of 9 days. Therefore the loss of color was greater (Fig. 1b) and occurs a variation from orange (less redness) to yellow (great yellowness). Then the MCNs were able to withstand high temperature but did not endure the exposure to accelerated conditions of light for a long time. The light exposure led to the same variation in CIELab parameters during storage of bixin microcapsules (De Marco, Vieira, Ugri, Monteiro, & Bergamasco, 2013).

The ΔE value denotes a difference in color between two samples, in the range of 0 to 0.5 signifies an imperceptible difference in color, 0.5 to 1.5 a slight difference, 1.5 to 3.0 a just noticeable difference, 3.0 to 6.0 a remarkable difference, 6.0 to 12.0 an extremely remarkable difference, and above 12.0 a color of a different shade (Kim et al., 2002), it means that a high ΔE implies a significant color variation. For Isotonic-NC, the difference in ΔE values between samples at zero time and 30 min of storage were less than 3.0, but before 60 min there was a high variation 15.19 and 14.73 in dark or light respectively (Table 2), consequently from this time the samples showed a different shade color. As was expected in light exposure the final ΔE value was higher, it means a significant loss of quality color. These results demonstrate that decoloring by light exposure progressed rapidly from 30 min of storage. Otherwise, the difference in ΔE values for Isotonic-MCN samples was less than 3.0 during all storage time in the absence of light, while for samples stored on light exposure from 9 days the exhibited a remarkable difference and after 15 days was considered extremely remarkable (Table 2). Mesnier et al. (2014) reported that $\Delta E > 2$ is used as a threshold value to indicate that the shade has started to change indicating a loss of color visually. A difference of less than this threshold value is considered unnoticeable to the naked eye. Thus, these findings show that the storage light condition is a factor that has an important role in the stability of the color in the beverages. It can also be observed that the coating material offers better protection from decoloring at a high temperature of storage evaluated since lower differences during the storage time were obtained.

Several characteristics that permits assess the color stability such as stronger saturation, more vivid color, and less yellowness are considered important to keep the quality of tangerine beverages rendering the interesting in achieving an acceptable C^* and h°_{ab} values according to with CIELAB color chart for the desired orange tonality,

besides of low ΔE^* . There were significant differences ($p < 0.05$) of C^* and h°_{ab} values during storage of Isotonic-NC and Isotonic-MCN (Table 2.), but there was a slight decrease of C^* when samples of both beverages were stored in the absence of light and little h°_{ab} increase, it means a change from orange to yellow. In contrast, when both were exposed to light, these showed a high loss in saturation. Therefore the final samples showed a different shade color. C^* and h°_{ab} depends on a^* and b^* , thus was possible to determinate that light influences the degradation of these parameters.

3.2.2 Kinetic behavior during storage

Accelerated conditions of storage (heat and light) result in a compound loss over time, and such effect was observed for both, Isotonic-NC and Isotonic-MCN (Fig. 2). As was expected during all kinetic reactions the behavior of norbixin thermal degradation was of first-order and kinetic degradation constants (k_t) was calculated through an exponential regression fit for Isotonic-NC and Isotonic-MCN in absence or light exposure with correlation factors ($0.94 > R^2 > 0.98$). The fast decay of norbixin retention (%) in Isotonic-MCN (Fig. 2b) at the beginning of the heating test may be due to the degradation of non-encapsulated compound located on surface of microcapsules and consequently exposed directly to heat and light conditions (Barbosa et al., 2005; Lobato et al., 2015).

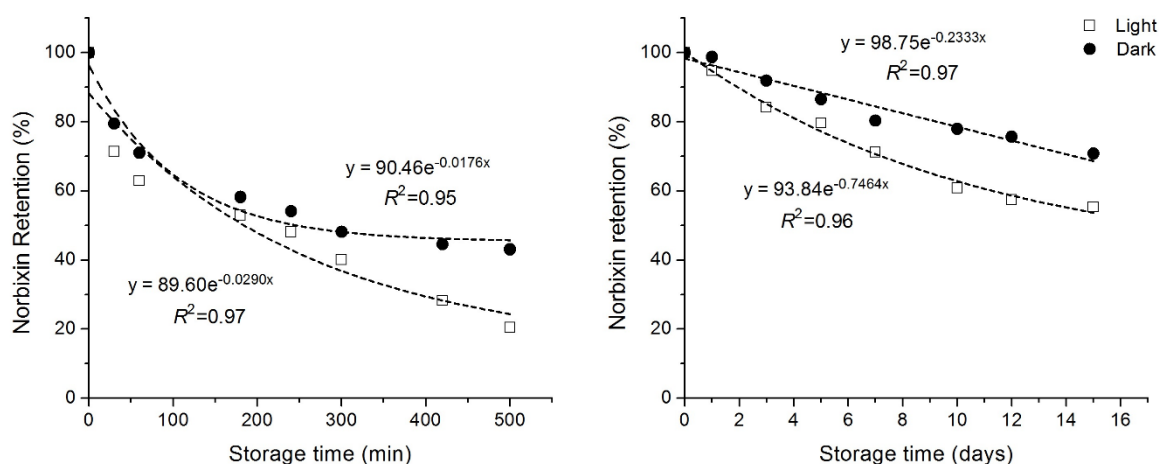


Fig.2. Norbixin retention (%) during storage on accelerated conditions a) Isotonic tangerine soft drinks samples prepared with norbixin crystals (Isotonic-NC) at 35°C, b) Isotonic tangerine soft drinks samples prepared with norbixin microcapsules (Isotonic-MCN) at 40°C.

For each storage conditions, the $t_{1/2}$ parameter was calculated with k_t value in order to predict the time required for norbixin to reduce a 50% of its initial measurement. As it can be observed, $t_{1/2}$ depends on light exposure, and for all experiments, $t_{1/2}$ was

higher on the dark (Table 3.). In the presence of light, the $t_{1/2}$ was smaller due to the use of accelerated treatments that are inducing a faster degradation of compounds.

When was evaluated the norbixin stability of Isotonic-NC samples in the absence of light the result of $t_{1/2}$ was greater than the $t_{1/2}$ value on the light. This behavior means that norbixin retention decrease was influenced by light exposure and isotonic beverages coloring with free norbixin are not able to maintain their quality for a long time even in the dark. Approximately 3 hours were enough to a significant loss of norbixin (Fig. 2a).

Table 3. Rate kinetic parameters: degradation constant (k_t) and half-life time ($t_{1/2}$) for non-encapsulated system (Isotonic-NC) and isotonic tangerine soft drink with norbixin microcapsules (Isotonic-MCN).

Sample	Isotonic-NC		Isotonic-MCN		
	Dark	Light	Dark	Light	
Rate parameters					
$k_t \times 10^{-3} \text{ (min}^{-1}\text{)}$	1.76	2.90	$k_t \times 10^{-3} \text{ (day}^{-1}\text{)}$	23.33	74.64
$t_{1/2} \text{ (min)}$	393.39	238.93	$t_{1/2} \text{ (days)}$	29.71	9.27

(k_t) degradation constant; ($t_{1/2}$) half-life time

(Isotonic-NC: Isotonic tangerine soft drink with norbixin crystals, Isotonic-MCN: Isotonic tangerine soft drink with norbixin microcapsules)

For Isotonic-MCN samples, the result of $t_{1/2}$ of norbixin was extremely higher in comparison with Isotonic-NC (Table 3.). Thus, confirming that encapsulation technique using GA as wall material is an alternative to enhance a shelf life of bioactive compounds. When norbixin is non-encapsulated is more sensible to degradation. Therefore norbixin loss from Isotonic-NC occurs more rapid than from Isotonic-MCN, showing the encapsulated compound has upper resistance to high temperatures. When the use of GA as a coating material to carry out microcapsules by spray-drying was compared to other polymers (maltodextrin, whey protein isolate, modified starch or their blends) increases $t_{1/2}$ and significantly influences the increase in shelf life of bioactive compounds (Al-Ismael, El-Dijani, Al-Khatib, & Saleh, 2016; Kanakdande, Bhosale, & Singhal, 2007). The good emulsifying properties and the ability of this carbohydrate to form a film around of interest compound are responsible for a good retention and protection on accelerated conditions of storage, thus offering a plasticity property that prevents the cracking of the protection matrix (Krishnan, Kshirsagar, & Singhal, 2005). The chemical structure of GA presents molecules of arabinogalactan protein (AGP), the properties of this compound offers the emulsifying characteristics of water and good capability film forming of this polymer (Castellani et al., 2010). In agreement with Barbosa, Borsarelli, & Mercadante

(2005) that reported an enhanced stability of bixin when was encapsulated with GA compared to other polymers and greater stability was noted between microcapsules stored at light exposure than those stored in the dark. The stability was higher for encapsulated bixin compared to free bixin. Cano-Higuita, Malacrida, & Telis (2015), studied the curcumin stability during storage at 25°C under light and reported that encapsulating matrix containing GA led to the high retention of curcumin when spray-drying was used as the drying method. In a study carried out by Guan & Zhong (2014), was founded that GA inhibited the hydrolysis of norbixin structure efficiently during heating. Additionally, this polymer prevents the norbixin precipitation in acidic conditions (pH = 3.0 – 4.0).

Therefore, encapsulation process with GA provided an enhanced stability of norbixin at the studied conditions of heat and light in a food matrix during storage. This agrees with reports that GA as coating material can effectively improve the stability of bioactive compounds such as carotenoids (Barbosa et al., 2005; Bustos-Garza et al., 2013; Cano-Higuita et al., 2015; De Marco et al., 2013; Lim et al., 2016), anthocyanins (Burin, Rossa, Ferreira-Lima, Hillmann, & Boirdignon-Luiz, 2011; Chung, Rojanasasithara, Mutilangi, & McClements, 2016; Pitalua, Jimenez, Vernon-Carter, & Beristain, 2010) and essential oils (Alves et al., 2014; Gallardo et al., 2013; Kanakdande et al., 2007).

Consequently, in this study was demonstrated that norbixin is more stable during storage on accelerated conditions when is encapsulated than it is exposed in free form because the losses were faster in Isotonic-NC (hours) when was compared with Isotonic-MCN that needed more time (days) and higher temperature (Table 3).

Accelerated treatments with heat and light exposure are commonly conducted in stability studies of bioactive compounds to perform a shelf life estimation. The thermal stability of free norbixin applied as natural dye in an acidified aqueous solution (orange juice ready to serve) was evaluated by Prabhakara Rao, Satyanarayana, & Rao (2002) using refrigeration temperature (7 - 10°C) and room temperature (25 - 30°C) to simulate storage conditions for a long period (5 months). At room temperatures, the authors reported a significant loss in norbixin concentration and color attributes, for low temperatures the degradation losses were not significant. Gallardo-Cabrera & Rojas-Barahona (2015) carried out a stability study of an aqueous solution of norbixin during storage at 30°C under light and reported the highest shelf life. Nonetheless, the results obtained in these research could not compare directly with results of this study because

the authors used different concentrations and temperatures, besides of performing the samples storage in containers without direct transmission of light.

Isotonic-MCN samples stored in the absence of light needs approximately 3 times more storage days than samples stored at light exposure to achieve a 50% of norbixin loss (Table 3.). The samples showed 70% of norbixin retention before 15 storage days. Whereas that samples stored in the light before the ten days of storage exhibited a high degradation (Fig. 2b) in concordance with the color loss at the same time (Fig. 1b.). The influence of light on norbixin stability was more significant than the effect of heating. Parvin, Aziz, Yusof, Sarker, & Sill, (2011) and De Marco et al. (2013), studied the stability during storage of norbixin extracts and bixin microcapsules, respectively. The authors reported a higher $t_{1/2}$ in the dark than under light at room temperature in the model aqueous system. Burin, Rossa, Ferreira-Lima, Hillmann, & Boirdignon-Luiz (2011), studied the stability of anthocyanins microcapsules applied as a natural dye in isotonic soft drinks systems and reported the same behavior.

The carotenoids are prone to isomerization and oxidation during processing and storage of foods due to their chemical structure. The main cause of carotenoid loss is enzymatic or non-enzymatic oxidation, which depends on the availability of oxygen, structure of the carotenoid and type of food matrix. Light, heat, acids, metals, enzymes and peroxides promote it. The length of storage on severe conditions (temperature, moisture, light), as well as the use of packaging permeable to oxygen and light, increases the degradation and decreases the shelf life of processed foods (Rodriguez-Amaya, Rodriguez, & Amaya-Farfan, 2006).

The addition of ascorbic acid (AA) could be a factor that negatively influences the degradation of norbixin. The impact of AA on light and heat stability of carotenoids is beneficial only at high concentrations, at a low concentration, a pro-oxidant effect could occur (Mesnier et al., 2014). The enrichment of beverages with AA could promote the isomerization of carotenoids and contact with organic acids during their deterioration (Meléndez-Martínez, Vicario, & Heredia, 2009). To date, only anthocyanins have been applied as a natural dye in isotonic soft drink systems and were evaluated the color and stability during storage under light or in the dark. As was expected, the effect of light was harmful to all experiments, but was not statistically significant ($p > 0.05$) for the acerola systems due to the high AA levels naturally present in the acerola anthocyanin extracts, since direct condensation reaction between AA and anthocyanins can occur in all acerola systems (de Rosso & Mercadante, 2007). When was assessed the stability of

anthocyanins as a natural dye in model beverages, the authors reported that in the presence of AA the stability of anthocyanin was reduced and showed extensive color loss during storage due to hydrogen peroxide formed through ascorbic acid oxidation. Through the addition of low levels of GA was possible to improve stability, this result was attributed to the interaction between anthocyanin and GA through hydrogen bonding. In this way, the anthocyanin achieves a barrier against AA condensation or oxidation by hydrogen peroxide during storage (Chung et al., 2016). Based on the results of this study it could be suggested that a higher concentration of GA could increase the beneficial interaction between AA and GA, which causes a decrease in the pro-oxidant effect of AA, thus an enhanced light stability of norbixin when applied in beverages systems as a natural colorant.

4 Conclusion

In this study, through the application of norbixin microcapsules obtained by *spray-drying* with gum arabic was possible to reach the desired orange color of an isotonic tangerine soft drink, as well as determined the kinetics rates of degradation during storage. The half-life time required for degradation of norbixin encapsulated was extremely high (29.71 days at 40°C) compared to free norbixin (6.56 hours at 35°C) under heating. The results show that the light exposure results in a decrease of norbixin retention in beverages than was stored in the dark. The addition of ascorbic acid promotes the norbixin deterioration. Nevertheless addition of gum arabic could prevent their pro-oxidation effect. Therefore, it was proven that encapsulation process is an excellent alternative to enhance the stability of norbixin in the aqueous model system and thus extend their shelf life. Overall, this study validated the potential use of norbixin microcapsules as a natural colorant to be applied in food matrices, especially beverages due to their hydro-solubility. The food and beverage industry can be used this information to development products with natural dyes instead of synthetic dyes as a contribution to human health.

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CAPITULO 4

4 DISCUSSÃO GERAL

Cada vez mais existe um aumento do interesse dos consumidores para adquirir alimentos que sejam saudáveis, portanto as indústrias de alimentos tentam substituir o uso de aditivos sintéticos em alimentos processados. A norbixina é um carotenoide hidrossolúvel que tem sido utilizado como corante natural em diferentes tipos de alimentos, porém é susceptível a degradação por exposição a fatores ambientais como luz, oxigênio e alta temperatura, e isso limita as suas aplicações. Até hoje a norbixina não foi usada como aditivo em bebidas, o qual pode representar uma excelente alternativa para aproveitar a sua hidrossolubilidade. Com base nisso, o presente trabalho desenvolveu uma possibilidade de melhorar a solubilidade e aumentar a estabilidade da norbixina em condições de processamento e armazenamento aplicado em uma bebida através da utilização do microencapsulamento com a técnica de secagem por atomização.

Em cada etapa do trabalho foi atingido um objetivo específico que permitiu estabelecer parâmetros para as seguintes avaliações e possíveis aplicações do produto obtido.

A norbixina foi extraída das sementes de urucum até conseguir cristais de norbixina com 100% de pureza. Após, para escolher qual o melhor material que poderia ser usado como agente de encapsulação da norbixina, foi decidido usar a eficiência de microencapsulação como referência devido ao interesse de manter a maior quantidade do composto bioativo no interior da microcápsula. A partir do levantamento bibliográfico foi possível observar que existem diferentes materiais de parede que já foram usados para encapsular carotenoides e que cada material tem vantagens e desvantagens que limitam a sua aplicação; mas, apesar disso, a maioria demonstrou que é capaz de melhorar a estabilidade dos carotenoides. Para esta nova etapa do estudo foram escolhidos a maltodextrina, goma arábica e a combinação destes materiais pelas características emulsificantes e a capacidade de formar filme em torno do composto encapsulado. O uso de 100% de goma arábica demonstrou ser mais eficiente na microencapsulação da norbixina com uma eficiência de microencapsulação de até 74,91% quando foi comparada com o uso de 100% de maltodextrina e as diferentes combinações desses carboidratos como materiais de parede, apresentando uma

diferencia estatística com todas as outras formulações que foram usadas. A solubilidade em água das microcápsulas obtidas foi maior que 90%.

Os resultados do estudo indicaram que foi possível encapsular cristais purificados de norbixina usando carboidratos como material de parede, e as microcápsulas obtidas mostraram uma boa homogeneidade em relação a distribuição do tamanho de partícula além de apresentarem atividade antioxidante, com alto potencial para ser aplicado em uma matriz alimentar como corante natural. Devido a esses resultados promissores acerca do microencapsulamento da norbixina, se constatou a necessidade de avaliar a estabilidade das microcápsulas em condições que mimetizam processamento e armazenamento, já que as reações de degradação são influenciadas por muitos fatores como calor, iluminação e presença de oxigênio.

A partir da melhor formulação obtida no primeiro estudo foram desenvolvidas microcápsulas de norbixina para avaliar sua estabilidade térmica em sistemas modelo aquoso. A energia de ativação (E_a) requerida para a degradação das microcápsulas de norbixina ($E_a = 15,08$ kcal/mol) foi o dobro do que para norbixina não encapsulada ($E_a = 7,61$ kcal/mol). Portanto, foi demonstrado que o processo de microencapsulamento indica uma alternativa eficiente para melhorar a estabilidade da norbixina quando exposta às altas temperaturas. Com base nisso, as microcápsulas de norbixina poderiam resistir processos térmicos quando adicionadas em alimentos processados e dessa forma ter uma maior vida de prateleira.

Na última etapa da pesquisa as microcápsulas com as melhores características (100% goma arábica) foram aplicadas em uma bebida com o fim de aproveitar sua alta solubilidade em água e avaliar sua estabilidade sob condições aceleradas de armazenamento. Para isso foi desenvolvida uma bebida isotônica de tangerina e foram aplicadas microcápsulas de norbixina como corante natural.

Foi possível obter a coloração desejada (laranja) com uma baixa concentração de norbixina ($2,86 \pm 0,02$ μg norbixina/mL). A bebida isotônica adicionada de microcápsulas de norbixina apresentou um tempo de vida média muito maior (29,71 dias) do que a bebida isotônica com norbixina não encapsulada (6,56 horas). O estudo demonstrou que a adição de microcápsulas de norbixina afeitou a estabilidade das bebidas favoravelmente durante o armazenamento. A adição de ácido ascórbico nas bebidas promove a deterioração da norbixina, porém, a adição de goma arábica poderia prevenir o efeito pro-oxidante do ácido ascórbico.

As microcápsulas desenvolvidas neste trabalho mostraram que o encapsulamento conferiu uma melhor solubilidade à norbixina e aumentou a sua estabilidade térmica quando exposto às altas temperaturas de processamento, assim como em diferentes condições de armazenamento tais como aquecimento, luz e oxigênio. Portanto, o microencapsulamento pode permitir o desenvolvimento de um corante natural com alto potencial para ser adicionado em alimentos processados para substituir o uso de corantes sintéticos.

5 CONCLUSÕES

Durante o processo de extração foi possível atingir um 100% de conversão de bixina para norbixina através da saponificação, e mediante a cristalização foram obtidos cristais de norbixina com 100% de pureza no comprimento de onda utilizado, sem formação de isômeros ou outros compostos, por tanto não houve degradação. Os melhores resultados na microencapsulação da norbixina foram obtidos quando a goma arábica foi utilizada como agente de encapsulação em comparação com a maltodextrina. Foi obtida uma alta eficiência de microencapsulação que provocou uma manutenção da atividade antioxidante da norbixina no interior das microcápsulas. Os resultados obtidos neste estudo comprovam a potencialidade da utilização de goma arábica na produção de microcápsulas de norbixina por suas propriedades emulsificantes em baixas concentrações. A energia de ativação requerida para a degradação da norbixina foi maior (dobro) para as microcápsulas do que para a norbixina livre quando ambas foram comparadas usando o mesmo sistema modelo. Portanto, o microencapsulamento ofereceu um aumento na estabilidade da norbixina quando exposta ao calor. A aplicação de uma pequena concentração de norbixina em bebidas isotônicas de tangerina permitiu obter a coloração desejada, portanto só foi necessário adicionar uma baixa quantidade de microcápsulas de norbixina. As bebidas isotônicas adicionadas de microcápsulas de norbixina mostraram um alto tempo de vida média sob condições aceleradas (aquecimento e luz) de armazenamento. Foi comprovado que o processo de microencapsulação é uma excelente alternativa para melhorar a estabilidade da norbixina em um sistema modelo aquoso e assim estender a sua vida de prateleira. Sobretudo, esse trabalho validou o potencial uso das microcápsulas de norbixina como corante natural para ser aplicado em diferentes matrizes alimentícias, especialmente em bebidas, devido à sua alta solubilidade em médio aquoso.

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