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JÉSSICA THAÍS DO PRADO SILVA

# NANOENCAPSULAÇÃO DE LUTEÍNA E AVALIAÇÃO *IN VIVO* DA SUA INFLUÊNCIA SOBRE A MEMÓRIA

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CAMPO MOURÃO
2017

JÉSSICA THAÍS DO PRADO SILVA

# NANOENCAPSULAÇÃO DE LUTEÍNA E AVALIAÇÃO *IN VIVO* DA SUA INFLUÊNCIA SOBRE A MEMÓRIA

Dissertação apresentada como requisito parcial para obtenção do título de Mestre em Tecnologia de Alimentos, do Programa de Pós-Graduação em Tecnologia de Alimentos – PPGTA – da Universidade Tecnológica Federal do Paraná/UTFPR, Campus Campo Mourão.

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Co-orientador: Dr. Gustavo Petri Guerra

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**NANOENCAPSULAÇÃO DE LUTEÍNA E AVALIAÇÃO IN VIVO DA SUA INFLUÊNCIA SOBRE A MEMÓRIA**

Por

**JÉSSICA THAÍS DO PRADO SILVA**

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* A via original com as assinaturas encontra-se na secretaria do programa.

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## RESUMO

Os carotenoides compõe um grupo de pigmentos lipossolúveis que apresentam inúmeros benefícios à saúde humana. Dentre eles, a luteína, uma xantofila de coloração amarelo-alaranjado, destaca-se devido à sua atividade biológica. Uma vez que o organismo humano é incapaz de sintetizar carotenoides, a aquisição de luteína é possibilitada apenas pela alimentação ou suplementação. Dietas ricas em luteína tem sido associadas à melhora da função cognitiva em adultos, contudo a estrutura química da luteína que proporciona seus efeitos benéficos é extremamente sensível ao calor, baixo pH, oxigênio dissolvido e exposição a luz. A encapsulação é uma técnica que tem se mostrado eficiente na proteção de compostos bioativos contra esses agentes externos e no aumento da biodisponibilidade no organismo, permitindo que tais compostos cheguem ao sítio de absorção. Este trabalho teve como objetivos obter e caracterizar luteína nanoencapsulada em polímeros biodegradáveis, realizar a validação de um método analítico para a determinação de luteína encapsulada por espectrofotometria UV-Vis, bem como avaliar seu efeito na memória declarativa de camundongos. Luteína foi encapsulada com êxito em matrizes de zeína e poli(vinil pirrolidona) separadamente, possibilitando a obtenção de partículas nanométricas e de formato esférico. Na primeira parte do trabalho, um método analítico para a determinação de luteína em nanopartículas de zeína foi validado, apresentando limite de quantificação de 4,407 $mg.L^{-1}$, parâmetro suficiente para calcular a eficiência de encapsulação em sistemas nanopartículados. Na segunda parte do trabalho, a encapsulação da luteína em nanopartículas de PVP foi capaz de aumentar em 43 vezes a solubilidade da luteína em água. Sua administração oral em camundongos (10 $mg.Kg^{-1}$ e 1,5 $mg.Kg^{-1}$), durante 14 dias apresentou efeito 66 vezes maior que a luteína livre na memória declarativa durante a tarefa de reconhecimento de objetos. A técnica se mostrou uma alternativa viável para aumentar sua solubilidade em água e, consequentemente, sua biodisponibilidade, contribuindo com o papel da luteína no comprometimento cognitivo.



**Palavras-chave:** Carotenoides. Encapsulação. Biodisponibilidade. Neuroproteção. Déficit cognitivo. Reconhecimento de objetos. Memória. Validação analítica.

## ABSTRACT

Carotenoids are a group of fat-soluble pigments presenting numerous benefits to human health. Among them, lutein, a yellow-orange xanthophyll, has attracted attention due its biological activity. Since the human body is unable to synthesize carotenoids, lutein acquisition is only possible through food or supplementation. Lutein rich diets have been associated with the improvement of cognitive functions in adults. However, the chemical structure of lutein, which is responsible for its beneficial effects, is extremely sensitive to heat, low pH, dissolved oxygen and exposure to light. Encapsulation is a technique that has proved effective in protecting bioactive compounds against external agents and increased bioavailability in the body, allowing such compounds to reach its absorption site. This study aimed to obtain and characterize lutein nanoencapsulated in biodegradable polymers, perform the analytical validation of a UV-Vis procedure to determine the lutein concentration in the nanoparticles and to evaluate the effect of encapsulated lutein on declarative memory of mice. Lutein was successful encapsulated in zein and polyvinylpirrolidone matrix, separately, allowing obtaining nanometric and spherical particles. In the first part of this work, an analytical procedure for lutein determination in zein nanoparticles was validated presenting quantification limit of 4.407 $mg.L^{-1}$, which is a sufficient parameter to calculate entrapment efficiency in nanoparticulate systems. In the second part of this work, lutein encapsulation in PVP nanoparticles was able to increase 43 times lutein water solubility and its oral administration to mice (10 $mg.Kg^{-1}$ and 1.5 $mg.Kg^{-1}$), during 14 days presented effect 66 higher than pristine lutein on mice's declarative memory during object discrimination task. This technique showed to be a viable alternative to increase lutein water solubility and, consequently, its bioavailability, contributing with lutein's role in cognitive impairment.



**Keywords:** Carotenoids. Encapsulation. Bioavailability. Neuroprotection. Cognitive Deficit. Object recognition. Memory. Analytical validation.

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## 1 INTRODUÇÃO

Os carotenoides são pigmentos orgânicos lipossolúveis sintetizados geralmente em plantas superiores, algas e bactérias. Esses pigmentos apresentam um grupo variado de compostos valiosos para a área farmacêutica, química e indústrias de alimentos, não somente por alguns deles serem precursores da vitamina A, mas também pela sua potente capacidade antioxidante e efeito corante (SANT'ANNA et al., 2013). São divididos em duas classes, carotenos e xantofilas e, dentre as xantofilas, destaca-se a luteína ($C_{40}H_{56}O_2$), um pigmento de coloração amarelo-alaranjada (GONNET; LETHUAUT; BOURY, 2010). Animais não são capazes de sintetizar luteína, dependendo exclusivamente da ingestão de alimentos ricos nesse composto, como frutas e vegetais, especialmente vegetais de folhas verdes.

Dentre o conjunto de atividades biológicas que a luteína apresenta, ela tem sido associada à melhora da função cognitiva em adultos (ARUNKUMAR; PRASHANTH; BASKARAN, 2013). Uma das principais causas do comprometimento cognitivo é o aumento do estresse oxidativo no cérebro. O cérebro é um órgão propenso ao ataque de oxigênio devido ao seu conteúdo antioxidante relativamente baixo, teor considerável de ácidos graxos poliinsaturados presentes na membrana neural e alto consumo de oxigênio. Deste modo, a hipótese de que os carotenoides possuem efeito protetivo nas funções cognitivas é baseada em suas habilidades de interceptar os radicais peroxil e inibir moléculas de oxigênio singleto, o que lhes permite evitar a peroxidação lipídica (AKBARALY et al., 2007).

Contudo, sua estrutura química, que é responsável por suas propriedades antioxidantes, faz com que esse pigmento seja susceptível à degradação por agentes externos, como calor, baixo pH, oxigênio dissolvido e exposição a luz (SANT'ANNA et al., 2013).

A técnica de encapsulação é um procedimento que vem sendo empregado com a finalidade de reduzir e/ou prevenir os processos de degradação até que os compostos cheguem aos sítios onde sua absorção é desejada (DE VOS et al., 2010). Trata-se de uma tecnologia onde o composto bioativo é envolto por uma barreira física. Além disso, essa técnica possibilita o empacotamento de sólidos, líquidos ou gás em pequenas cápsulas que liberam o seu conteúdo por períodos

prolongados em condições especificas, aumentando a biodisponibilidade do composto encapsulado (NEDOVIC et al., 2011).

A eficiência de encapsulação é uma das principais análises de caracterização de sistemas nanoparticulados. Essa técnica exige a quantificação adequada do composto encapsulado, de modo a verificar a quantidade que realmente foi aprisionada dentro das partículas. Deste modo, a validação de um método analítico para a quantificação de luteína é uma maneira adequada de garantir a confiabilidade e comparabilidade dos resultados.

Diversos estudos indicam que a técnica de encapsulação é capaz de aumentar a biodisponibilidade de luteína *in vitro* e *in vivo* (ARUNKUMAR et al., 2015; ARUNKUMAR; PRASHANTH; BASKARAN, 2013; KAMIL et al., 2016; LACATUSU et al., 2013; LIU; WU, 2010; QV; ZENG; JIANG, 2011; STANCANELLI et al., 2012; ZHAO et al., 2014). Entretanto, a avaliação da influência da luteína encapsulada na função cognitiva *in vivo* é um assunto escasso na literatura científica.

Nesse contexto, a encapsulação da luteína em materiais biodegradáveis e/ou biocompatíveis se apresenta como uma alternativa promissora para melhorar suas propriedades tecnológicas e aumentar sua biodisponibilidade, contribuindo com o papel da luteína no comprometimento cognitivo.

## 2 APRESENTAÇÃO

A presente Dissertação foi estruturada da seguinte forma. Primeiramente, uma breve introdução ao tema e os objetivos são apresentados. Depois, uma revisão bibliográfica, sem o intuito de esgotar o tema é apresentada no Capítulo 4 sobre a luteína, sua encapsulação e seus efeitos biológicos, com ênfase nas funções cognitivas. No Capítulo 5, uma técnica analítica para a determinação da eficiência de encapsulação de luteína em zeína é validada e aplicada para nanopartículas de zeína contendo luteína. Logo após, a nanoencapsulação da luteína em poli(vinil pirrolidona) é realizada no Capítulo 6, sendo as nanopartículas submetidas a diversas técnicas de caracterização. Nesse capítulo também são apresentados os resultados em relação à influência da luteína encapsulada na memória declarativa de camundongos. Finalmente, no Capítulo 7 são apresentadas as conclusões gerais desta dissertação.

# 3 OBJETIVOS

## 3.1 Objetivo Geral

Obter nanopartículas de luteína em zeína ou em poli(vinil pirrolidona) e avaliar o efeito de nanopartículas luteína-poli(vinil pirrolidona) sobre a memória declarativa de camundongos.

## 3.2 Objetivos Específicos

- Obter nanopartículas de zeína contendo a luteína pela técnica de nanoprecipitação;
- Validar o método analítico para a determinação quantitativa de luteína nas nanopartículas zeína por Espectroscopia UV-Vis;
- Caracterizar as nanopartículas de zeína em relação ao tamanho, morfologia e eficiência de encapsulação;
- Obter nanopartículas de poli(vinil pirrolidona) contendo luteína pela técnica de nanodispersão;
- Caracterizar as nanopartículas de PVP em relação ao tamanho, morfologia, propriedades térmicas e químicas e solubilidade em água;
- Avaliar os efeitos da administração oral de luteína encapsulada sobre a memória de camundongos na tarefa de reconhecimento de objetos.

# 4 REVISÃO BIBLIOGRÁFICA

## 4.1 Compostos bioativos em alimentos

Os compostos bioativos são componentes de alimentos naturais que promovem efeitos farmacológicos em seres humanos e animais. Tipicamente, os compostos bioativos são metabólitos secundários de organismos vivos. Trata-se de um grupo de substâncias que os auxiliam a aumentar suas habilidades de sobreviver e superar mudanças locais, interagindo com os organismos que os rodeiam (AZMIR et al., 2013; MARSANASCO et al., 2015)

O uso de compostos bioativos com a finalidade medicinal data desde o início da civilização, com o uso de plantas para curar enfermidades. O aumento do conhecimento em relação à dinâmica química da natureza de diversas moléculas bioativas impulsionou o progresso dessa aplicação na área farmacêutica, aditivos alimentícios e até mesmo pesticidas naturais (AZMIR et al., 2013). O consumo diário de compostos bioativos está relacionado com a melhora na saúde de seres humanos através da redução dos níveis de colesterol plasmático, aterosclerose aórtica adnominal e de fatores de risco cardiovascular (SANT'ANNA et al., 2013). Ainda, podem atuar como antioxidantes, mantenedores do balanço homeostático, prevenção de doenças crônicas, dentre outros benefícios (WALLACE et al., 2015).

Os exemplos mais comuns de compostos bioativos são os ácidos graxos essenciais (ômegas-3 e -6, por exemplo), tocoferol, flavonoides, polifenóis, fitoesteróis, vitaminas lipossolúveis e carotenoides (MARSANASCO et al., 2015).

## 4.2 Carotenoides

Carotenoides são pigmentos orgânicos lipossolúveis sintetizados geralmente em plantas superiores, algas e bactérias. Esses pigmentos apresentam um grupo variado de compostos valiosos para a área farmacêutica, química e indústrias de alimentos, não somente por alguns deles serem precursores da vitamina A, mas também pelo seu efeito corante e ações benéficas no organismo humano (SANT'ANNA et al., 2013). Dentre seus

efeitos biológicos, os carotenoides se destacam por serem antioxidantes, potenciadores do sistema imunológico, inibidores de mutagêneses e transformações, inibidores de lesões pré-malignas, blindagem na fóvea de primatas e quelantes de fluorescência não fotoquímicas (CASTENMILLER; WEST, 1998).

Em relação à aplicação industrial, os carotenoides têm importância na indústria de alimentos como corante natural. As ligações duplas conjugadas presentes em sua estrutura química absorve a luz dentro da região visível, fornecendo uma intensa coloração que varia do amarelo pálido ao laranja ou vermelho escuro (MATTEA; MARTÍN; COCERO, 2009). Mais de 700 carotenoides foram descritos na natureza, entretanto, nem todas as fontes naturais dos mesmos estão presentes na nossa dieta. Estima-se que apenas 40 carotenoides estão disponíveis para absorção, metabolização e uso no corpo humano (FERNÁNDEZ-GARCÍA et al., 2012).

As fontes dietéticas de carotenoides são principalmente vegetais e frutas amarelas e laranjas (cenoura, manga, tomate, pimenta e melão), vegetais verdes escuros (alface, espinafre, brócolis e couve) e na gema de ovo. Podem ser obtidas também a partir de fontes animais, como pássaros, peixes, crustáceos e insetos (AMORIM-CARRILHO et al., 2014). Os carotenoides são divididos em duas classes: carotenos, que não apresentam moléculas de oxigênio, e xantofilas, que são hidroxicarotenoides e, consequentemente, menos hidrofóbicos que os carotenos (GONNET; LETHUAUT; BOURY, 2010).

Na natureza, os carotenoides se apresentam em complexos proteicos, o que inibe sua absorção. Deste modo, muitos processos são requeridos para sua metabolização, incluindo a digestão adequada da matriz alimentar, formação de micelas lipídicas no intestino delgado, absorção pela mucosa intestinal e transporte para a circulação linfática ou portal (LOANE et al., 2008).

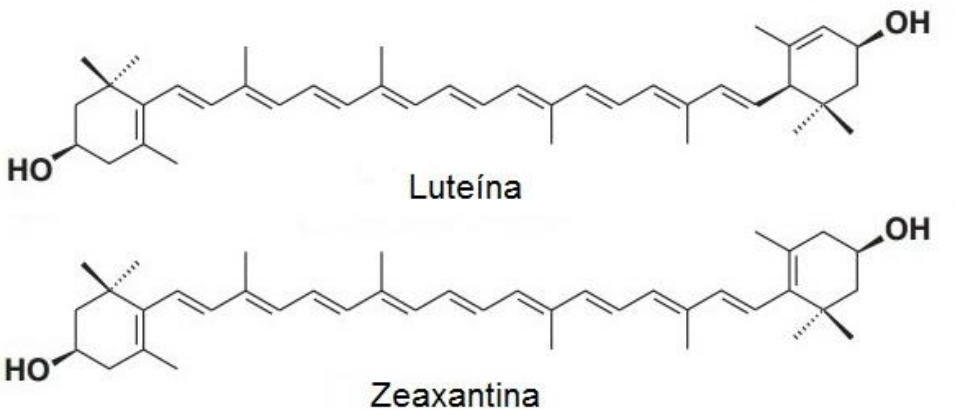
Partindo do princípio de que os seres humanos não podem sintetizar carotenoides, dependendo exclusivamente de fontes alimentícias para obter esses componentes e seus efeitos benéficos, sua biodisponibilidade sempre foi um assunto de interesse no meio cientifico (FERNÁNDEZ-GARCÍA et al., 2012). Em geral, a biodisponibilidade dos carotenoides depende da matriz alimentar, *status* de nutrientes do hospedeiro, fatores genéticos, competição entre nutrientes, incorporação de lipídios durante a refeição e conteúdo de fibra

alimentar ingerida. Alguns processos tecnológicos são capazes de aumentar a biodisponibilidade dos carotenoides, como processos térmicos de aquecimento e bioencapsulação (CASTENMILLER; WEST, 1998; NWACHUKWU; UDENIGWE; ALUKO, 2015).

### *4.2.1 Luteína*

A luteína é uma xantofila de fórmula molecular $C_{40}H_{56}O_2$, um pigmento amarelo-alaranjado da família dos carotenoides (HU et al., 2012). Este carotenoide se difere dos demais por possuir dois grupos hidroxilas, um em cada lado da molécula (WALLACE et al., 2015). Luteína e seu estereoisômero zeaxantina (Figura 1) se diferem apenas pela posição de uma dupla ligação no grupo hidroxila (ELDAHSHAN; IBRAHIM; EL-RAEY, 2013).

Figura 1 - Estruturas químicas da luteína e zeaxantina.




Fonte: Fernandez-Garcia et al. (2012).

Fontes dietéticas de luteína são folhas verdes, como espinafre, couve, repolho e outros alimentos, como milho, caqui, brócolis e gema de ovo (AMORIM-CARRILHO et al., 2014). Dentre todas as matrizes vegetais, as flores de calêndula mexicana (*Tagetes erecta* L.) possuem alto teor de luteína e, por esse motivo, são utilizadas como principal fonte de luteína purificada para aplicações tecnológicas. Nesta matriz, a luteína se encontra na forma de éster de ácido graxo, sendo necessárias várias etapas para a obtenção da luteína na forma livre. Os métodos convencionais consistem na extração dos

ésteres por meio de solvente orgânico ou $CO_2$ supercrítico, saponificação com solução alcalina e purificação (BOONNOUN et al., 2012).

Segundo Serpeloni et al. (2012), apesar de ser o segundo carotenoide mais encontrado na linfa humana, existem poucos estudos sobre os efeitos protetores causados pela luteína em relação aos outros carotenoides. A luteína é muito reconhecida por sua propriedade anti-inflamatória (BIAN et al., 2012; KIM et al., 2012; SELVARAJ; SHANMUGASUNDARAM; KLASING, 2010) e antioxidante (GAO et al., 2011; HAYASHI et al., 2014; WANG et al., 2013). Além disso, esse carotenoide tem sido considerado o principal recurso para a redução de danos oftalmológicos devido à Degeneração Macular Relacionada à Idade (DMRI) (LOANE et al., 2008; PENG et al., 2016). Ainda, a luteína tem sido associada à diminuição de danos ao fígado e intestino (DU et al., 2015; FLORES et al., 2014; LI et al., 2015b; SATO et al., 2011); prevenção da degradação do DNA (SERPELONI et al., 2012; WANG et al., 2006); prevenção de doenças cardiovasculares (KOH et al., 2011); redução do risco de câncer (LAKSHMINARAYANA et al., 2010); aumento da velocidade de processamento visual (BOVIER; HAMMOND, 2015) e, mais recentemente, estudos relacionam a luteína com efeitos neuroprotetores (AKBARALY et al., 2007; FEENEY et al., 2013; WOO et al., 2013).

#### 4.2.1.1 Luteína e funções cognitivas

Os benefícios de xantofilas têm sido bem estabelecidos para uma variedade de doenças e o seu potencial de melhorar funções neurológicas vêm emergindo na literatura (RENZI et al., 2014).

Dentre as principais causas do comprometimento cognitivo, o aumento do estresse oxidativo no cérebro se destaca. O cérebro é um órgão propenso ao ataque de radicais livres devido ao seu conteúdo antioxidante relativamente baixo, teor considerável de ácidos graxos poliinsaturados presentes na membrana neural e alto consumo de oxigênio (AKBARALY et al., 2007). O aumento da produção de radicais livres exerce efeitos nocivos às membranas fosfolipídicas, resultando na formação de produtos tóxicos à célula (ARNAL et al., 2010). De acordo com Finkel e Holbrook (2000), os radicais livres são gerados a partir do metabolismo intracelular que acontece na mitocôndria e

peroxissomos. Além disso, podem provir do recrutamento de células inflamatórias, que contém o potente sistema NADPH oxidase, produtor de grandes quantidades de superóxidos.

Luteína e seu isômero zeaxantina são os carotenoides predominantes no tecido cerebral humano, representando cerca de 66-77% da concentração total de carotenoides no cérebro. Apesar disso, as bases moleculares do efeito neuroprotor da luteína ainda é desconhecido, entretanto, alguns mecanismos tem sido propostos, como a diminuição do estresse oxidativo, ativação de vias anti-inflamatórias e modelação de propriedades funcionais de membranas sinápticas juntamente com mudanças em suas configurações físico-químicas e estruturais (JOHNSON, 2012). Ainda, de acordo com Erdeman Jr. e colaboradores (2015), a luteína localizada junto às membranas neurais pode influenciar na cognição através da manutenção da viabilidade celular, atuando na inibição de mecanismos de danificação, disfunção e morte celular.

A associação entre pigmento macular e funções cognitivas é um assunto recente na literatura científica. Feeney e colaboradores (2013) realizaram os primeiros trabalhos explorando a relação entre o pigmento macular e as funções cognitivas em humanos. Neste estudo, 4.453 adultos maiores de 50 anos de idade pertencentes ao Estudo Longitudinal Irlandês sobre Envelhecimento foram avaliados. Os resultados obtidos indicam que indivíduos com baixa concentração de pigmento macular, medidos com o auxílio de densiômetro métrico macular, apresentam memória prospectiva mais pobre, demoram mais tempo para completar tarefas de trilha e são mais lentos na tarefa de reação de escolha. Esses resultados sustentam a tese de que os pigmentos maculares podem ser uma ferramenta rápida e não invasiva para avaliar o aspecto de vulnerabilidade cognitiva na prática clínica, além de demonstrar que a suplementação nutricional de luteína e zeaxantina é capaz de fornecer maior grau de neuroproteção em indivíduos com níveis insuficientes desses carotenoides.

Vishwanathan et al. (2014) descreveram a distribuição de carotenoides em tecido cerebral de crianças. Vários carotenoides são detectados em regiões cerebrais associadas à memória, função executiva, visão e audição. Dentre todos os carotenoides, a luteína é o que apresenta maior concentração em todas essas regiões cerebrais. Ainda, os autores avaliam a influência dos

hábitos alimentares das crianças na concentração de luteína no tecido cerebral, onde os indivíduos que se alimentam por leite materno obtém maior concentração de luteína comparado aos que se alimentam por fórmulas infantis. Isso acontece porque a maioria das fórmulas infantis não são suplementadas com luteína, e quando são suplementadas, a luteína é menos biodisponível que a encontrada no leite materno. Essas são evidências substanciais para apoiar o papel da luteína no desenvolvimento precoce da visão e cognição.

O cérebro é organizado de modo que a memória seja uma função cognitiva separada e distinta das demais, podendo ser estudada isoladamente da percepção e outras habilidades intelectuais (BUFFALO; SQUIRE, 2015). Entre os tipos de memória existentes, a memória declarativa é aquela que promove acesso consciente a fatos e eventos e seu comprometimento ocorre quando estruturas do lobo temporal medial estão danificados (CLARKE; SQUIRE, 2010).

De acordo com Abbott (2011), o impacto econômico global causado por doenças neurodegenerativas em 2010 foi de 604 bilhões de dólares, valor que supera os custos com o câncer ou doenças cardíacas. Sendo assim, a forma mais rentável de combater essas patologias é através da prevenção, onde a intervenção nutricional se apresenta como principal estratégia (JOHNSON et al., 2013).

Apesar de todos os seus benefícios para a saúde humana, a luteína é instável ao calor, luz e oxigênio, devido suas oito duplas ligações conjugadas, além de possuir baixa solubilidade em água, o que torna o seu emprego tecnológico bastante limitado (ZHAO et al., 2014). Por esse motivo, é necessário utilizar técnicas capazes de estabilizar e proteger a luteína contra esses fatores externos.

### 4.3 Técnicas de encapsulação

Muitos compostos bioativos são altamente lipofílicos, ou seja, possuem baixa solubilidade em água, o que faz com que a adição desses compostos seja dificultada na maioria dos alimentos. Além disso, baixa solubilidade indica

baixa absorção no trato gastrointestinal, comprometendo a biodisponibilidade do mesmo (DONSÌ et al., 2011).

A técnica de encapsulação é um procedimento que vem sendo empregado com a finalidade de reduzir e/ou prevenir os processos de degradação até que o composto de interesse chegue aos sítios onde a sua absorção é desejada (DE VOS et al., 2010). Trata-se de uma tecnologia onde o composto bioativo é envolto por uma barreira física. Além disso, essa técnica possibilita o empacotamento de sólidos, líquidos ou gás em pequenas capsulas que liberam o seu conteúdo em taxas controladas por períodos prolongados em condições especificas (NEDOVIC et al., 2011).

Muitos procedimentos de encapsulação vêm sendo propostos por pesquisadores, mas nenhum deles pode ser considerado como método universal para aplicação em compostos bioativos. Cada composto bioativo possui características químicas particulares, que devem ser levadas em consideração para a escolha do método de encapsulação a ser utilizado. Um pré-requisito importante para o sistema de encapsulação é fornecer proteção ao composto bioativo de degradação química (oxidação ou hidrólise, por exemplo) e mantê-lo completamente funcional até o momento de sua absorção no organismo (DE VOS et al., 2010).

### *4.3.1 Encapsulação da luteína*

Diversas técnicas vêm sendo empregada para encapsular luteína: gelificação iônica (ARUNKUMAR; PRASHANTH; BASKARAN, 2013); transportador lipídico nanoestruturado (LACATUSU et al., 2013; LIU; WU, 2010); método de evaporação rotativa (ZHAO et al., 2014); emulsificação/evaporação do solvente (ARUNKUMAR et al., 2015; KAMIL et al., 2016); coacervação complexa (QV; ZENG; JIANG, 2011) e inclusão molecular (STANCANELLI et al., 2012). Independente da técnica utilizada, os resultados obtidos indicam que a técnica de encapsulação é capaz de aumentar a biodisponibilidade de luteína *in vitro* e *in vivo*. Entretanto, nenhum desses autores avaliaram a influência da luteína encapsulada na função cognitiva em estudos *in vivo*, concentrando-se em outras propriedades da luteína ou mesmo apenas na caracterização das partículas obtidas.

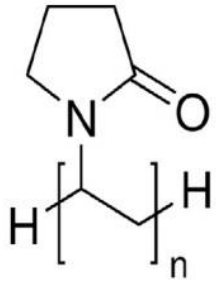
Dentre as técnicas de encapsulação, a nanoprecipitação (ou deslocamento de solvente) consiste na dispersão de gotas hidrofóbicas em um não-solvente. O soluto hidrofóbico (polímero ou molécula lipídica) é dissolvido em um solvente orgânico apolar. Em seguida, a solução é gotejada em um não-solvente do soluto (geralmente água), sob agitação. A mistura binária obtida se torna um não-solvente para as moléculas hidrofóbicas, levando à separação de fases que culmina na formação de partículas do soluto hidrofóbico. Em relação aos métodos de encapsulação à base de emulsão, a técnica de nanoprecipitação não requer o preparo de uma emulsão prévia, o que o torna uma opção mais rápida (LEPELTIER; BOURGAUX; COUVREUR, 2014a).

Nanopartículas de zeína, uma proteína vegetal extraída do milho (*Zea mays* L.), podem ser obtidas por nanoprecipitação. Sua estrutura é formada majoritariamente por aminoácidos não-polares, o que faz essa proteína ser insolúvel em água (PODARALLA; PERUMAL, 2012). A zeína é considerada um biopolímero versátil para a produção de nanopartículas e tem sido utilizada para a encapsulação de diversos compostos bioativos (CHEN; ZHONG, 2014). Além disso, o uso dessa proteína como material encapsulante aplicada a técnica de nanoprecipitação têm mostrado resultados satisfatórios (LI et al., 2013; PATEL et al., 2010; PATEL; BOUWENS; VELIKOV, 2010).

Outra técnica de encapsulação que tem se mostrado eficaz para aumentar a solubilidade de compostos lipofílicos é a nanodispersão. Neste método, o composto bioativo e polímero são adicionados em um solvente orgânico capaz de solubilizar as duas substâncias simultaneamente, seguido da diminuição do tamanho das partículas através de dispersores e, por fim, evaporação completa do solvente. O aumento da solubilidade de compostos lipofílicos causados pela técnica de nanodispersão é baseada principalmente em três mecanismos diferentes: molhabilidade do composto (que é melhorado através do contato direto com a matriz hidrofílica), redução do tamanho das partículas (aumentando a superfície de contato) e conversão do estado cristalino para o estado amorfo. Trata-se de um método viável e econômico para aumentar a biodisponibilidade de compostos pouco solúveis em água (FRIZON et al., 2013).

Um polímero promissor para uso na técnica de nanodispersão é o poli(vinil pirrolidona) (PVP, Figura 2), um polímero de fórmula molecular $(C_6H_9ON)_n$ pertencente ao grupo de lactama polimérica com amida interna. Este polímero se destaca por sua propriedade anfifílica e boa solubilidade em água e em solventes orgânicos convencionais, o que lhe confere alta biocompatibilidade, adesividade e alto poder em estabelecer ligações químicas com diversos compostos (SHPOTYUK et al., 2016). Por não ser tóxico, o PVP se apresenta como material promissor para aplicações em biotecnologia, indústrias farmacêuticas e cosméticas, além de já ser utilizado como estabilizante na indústria de alimentos (GUPTA; CHEN; LEE, 2015). O seu uso para o preparo de dispersões sólidas têm se mostrado promissor em relação ao aumento da solubilidade de compostos lipofílicos (FRIZON et al., 2013; KARAVAS et al., 2006).

Figura 2 - Estrutura monomérica do poli(vinil pirrolidona).




Fonte: Gupta; Chen e Lee (2015).

## 4.4 Validação analítica

Procedimentos analíticos que permitam a determinação precisa de eficiência de encapsulação são essenciais em sistemas nanoparticulados. A analise de dissolução é uma das técnicas mais utilizadas para a avaliação da eficiência de encapsulação, onde as partículas são desintegradas para liberar todo o seu conteúdo, seguido pela quantificação do composto encapsulado utilizando um método analítico apropriado (LEPELTIER; BOURGAUX; COUVREUR, 2014b; LÓPEZ; CALLAO; RUISÁNCHEZ, 2015). Por isso, a validação de métodos analíticos devem ser realizados a fim de garantir a

confiabilidade e comparabilidade dos resultados (LÓPEZ; CALLAO; RUISÁNCHEZ, 2015).

Atualmente, os métodos validados para determinar a eficiência de encapsulação de luteína utilizam Cromatografia Liquida de Alta Eficiência (ABRAHAMSSON; RODRIGUEZ-MEIZOSO; TURNER, 2012; CARERI; ELVIRI; MANGIA, 1999; DIAS; CAMÕES; OLIVEIRA, 2008; INDYK et al., 2014; KARLSEN et al., 2003; LI et al., 2015a, 2012a; MARINOVA; RIBAROVA, 2007; STINCO et al., 2014). Essas análises são caras e geram uma quantidade considerável de resíduos químicos. Os métodos espectroscópicos são mais baratos e utilizados com mais frequência em sistemas de encapsulação devido a sua rapidez e resultados confiáveis. Deste modo, vale a pena desenvolver e validar métodos utilizando espectroscopia na região ultravioleta-visível.

### 4.5 Desafios na encapsulação da luteína e sua aplicação

Apesar da luteína ter se demonstrado um agente eficaz na proteção de células cerebrais (AKBARALY et al., 2007; FEENEY et al., 2013; JOHNSON et al., 2013) e do reconhecimento da técnica de encapsulação como uma alternativa promissora no aumento de sua biodisponibilidade (ARUNKUMAR et al., 2015; ARUNKUMAR; PRASHANTH; BASKARAN, 2013; ZHAO et al., 2014), ainda não são retratados na literatura científica trabalhos que relatem o efeito da luteína encapsulada no cérebro. Deste modo, um estudo detalhado que relacione a influência da encapsulação nas propriedades neuroprotetoras da luteína, bem como a sua via de atuação nas células cerebrais se faz necessário. Assim, é preciso esclarecer qual a ação da luteína encapsulada no cérebro, inicialmente caracterizando as nanopartículas obtidas e avaliando sua eficácia *in vivo* em modelos animais. Esses dados devem servir de base para, futuramente, aplicá-los em pessoas que sofram de doenças neurodegenerativas.

# 5 ANALYTICAL VALIDATION OF AN ULTRAVIOLET-VISIBLE (UV-VIS) PROCEDURE FOR DETERMINING LUTEIN CONCENTRATION AND APPLICATION TO LUTEIN-LOADED ZEIN NANOPARTICLES

## 5.1 Introduction

Analytical procedures that allows precise determination of entrapment efficiency is essential in the encapsulation field. Dissolution assays is one of the most widely used techniques for entrapment efficiency evaluation in which particles are disintegrated to release the whole drug content followed by drug quantification using an appropriate analytical method. Validation of an analytical procedure must be carried out to guarantee reliability, traceability or comparability of results. Thus, developing and validating an analytical procedure for determination of lutein entrapment efficiency is extremely important. The objective of this work was to validate an analytical method based on Ultraviolet-visible Spectroscopy to determine the lutein concentration in lutein-loaded zein nanoparticles.

These results were submitted as a manuscript to Food Chemistry (ISSN 0308-8146).

## 5.2 Material and Methods

### *5.2.1 Material*

Lutein (90% purity, kindly gifted by Pincredit Bio-tech Co.), ethanol (99.5%, Neon), ethyl acetate (99.5%, Neon) and methanol (99.8%, Neon) were used as received. Zein (Sigma-Aldrich) was used as wall material. Distilled water and sodium caseinate (Sigma-Aldrich) were used as the continuous phase and stabilizing agent, respectively. Solutions were filtered through Millipore® nylon membrane filters (diameter 0.45 μm) and centrifuged in Amicon filters (100 kDa).

*5.2.2 Validation Procedure*

5.2.2.1 Specificity

Specificity is the ability to properly assess the analyte in the presence of components that may be present, such as impurities, degradants, matrices, among others (ICH, 2005). In order to demonstrate that no interference occurred due to the other nanoparticles constituents, specificity tests was carried out through the obtainment of the UV-Vis spectra of lutein and the zein nanoparticles without lutein (blank nanoparticles sample). Lutein was dissolved in methanol:ethyl acetate 1:1 (v/v) to yield a 240 $mg.L^{-1}$ solution. Then, 500 µL of the blank nanoparticles dispersion was centrifuged in Amicon filters during 30 minutes at 14,500 RPM. The filtrated was lyophilized (Liotop, L101) and then suspended in 1 mL methanol and 1 mL ethyl acetate. The solution was filtered (Millipore membrane filter, 0.45 µm) and analysed by UV-Vis spectrometry.

5.2.2.2 Linearity

Linearity is often checked in an analytical range by inspection of the correlation coefficient (r) of the calibration curve (Araujo, 2009; ICH, 2005). Linearity was investigated using lutein solutions in methanol and ethyl acetate 1:1 (v/v) in five different concentration levels ranging from 6 $mg.L^{-1}$ to 240 $mg.L^{1}$. Experiments were carried out in triplicate using UV-Vis at 446 nm.

5.2.2.3 Quantification Limit

Quantification limit (QL) is defined as the lowest amount or concentration of analyte that can be detected with an acceptable level of precision and accuracy (Araujo, 2009; ICH, 2005). QL was determined according to Equation 1, where b is the angular coefficient from the calibration curve and s is the standard deviation calculated from seven blank samples (no analyte added).

$$Q = s\ x\ \frac{10}{b} \quad (1)$$

5.2.2.4 Detection Limit

Detection limit (ICH, 2005) is determined by the analysis of samples with known analyte levels as stated in Equation 2, where s is the standard deviation from samples without analyte added and b is the angular coefficient of calibration curve equation (ICH, 2005).

$$D = s\ x\ \frac{3.3}{b} \quad (2)$$

5.2.2.5 Accuracy

Accuracy is a fundamental stage in method validation since it enables the assessment of trueness and precision of an analytical methods by means of concordance between reference value and obtained value (González, Herrador, & Asuero, 2010; ICH, 2005).

Accuracy was evaluated by the recovery method analysing three different concentrations of lutein solutions in methanol:ethyl acetate (1:1, v/v), within the previously determined standard calibration curve (6, 24 and 240 $mg.L^{-1}$). Samples were analysed by UV-Vis spectrometry in triplicate on two different days and the respective concentrations were recalculated from the calibration curve.

5.2.2.6 Precision

Precision was measured by repeatability (short period of time and one analyst), which is the best condition of an analysis; intermediate precision (different days and different analysts) that expresses the precision within-laboratory variations; and reproducibility (inter-laboratory studies) that represents precision between distinct laboratories. Precision of an analytical

procedure can be expressed as relative standard deviation percentage (RSD%) (ICH, 2005).

Repeatability, intermediate precision and reproducibility were determined with six scans of three lutein solutions at 6, 24 and 240 $mg.L^{-1}$. Precision levels were calculated through the RSD% from the analytical curves. UV-Vis spectra were acquired using an Ocean Optics equipment (model USB-650-UV-VIS Red Tide) in Laboratory of Food Analysis. The reproducibility analysis was performed by the same analyst in the Laboratory of Spectroscopy using a second equipment of the same model mentioned above.

5.2.2.7 Validation Equipment and Statistical Analyses

For the method validation an ultraviolet-visible spectrophotometer was used (Ocean Optics, USB-650-UV-VIS Red Tide) and scanning was performed from 200 to 850 nm with a 1 nm resolution.

ANOVA, adjusted determination coefficient and standard error of estimate were used to evaluate the calibration curve. The effects of concentration, day and their interaction were tested by factorial ANOVA. One-way ANOVA was implemented to determine the influence of laboratory and analyst. All analyses were carried out using Statistica 7.0 and a significance level (p) of 0.05 was considered.

*5.2.3 Preparation of the lutein-loaded zein nanoparticles*

Nanoparticles were produced by the nanoprecipitation technique according to Patel, Bouwens and Velikov (2010) with minor modifications as follows. Initially the organic phase composed by lutein (0.094 g) and zein (0.750 g) was prepared by dissolution in an ethanol:water solution (80:20 v/v, 30 mL) at 40$^{o}$C. After that, the aqueous phase was prepared by the dissolution of sodium caseinate (1.125 g) in distillate water (90 mL) at 60$^{o}$C. The aqueous phase was added under stirring at 10,000 RPM (Ultra Turrax IKA, model T25) while the organic phase was slowly dripped into the aqueous phase. The obtained suspension was allocated in a thermal bath with orbital shaker at 40oC for 24 hours to evaporate ethanol.

*5.2.4 Determination of the entrapment efficiency in the nanoparticles*

Total lutein concentration ($[lutein]_{total}$) was determined by the dissolution of 0.006 g lyophilized nanoparticles in 6 mL methanol followed by stirring during four hours. Then, zein was precipitated by adding 1 mL of ethyl acetate and the then filtered with a Millipore membrane (0.45 µm). Absorbance was determined at 446 nm by UV-Vis spectrometry (as described above).

To determine the amount of lutein inside the nanoparticles (entrapped lutein), 500 µL of the nanoparticles dispersion was immediately centrifuged in Amicon (100 KDa) filters during 30 minutes at 14,500 RPM and the obtained filtrate containing the free lutein ($[lutein]_{non\ entrapped}$) was lyophilized and diluted in 1 mL methanol. Zein was precipitated by adding 1 mL ethyl acetate, the solution was filtered with a Millipore membrane (0.45 µm) and the absorbance was determined at 446 nm. The entrapment efficiency (EE) was calculated according to Equation 3. This procedure was also carried out for nanoparticles produced without lutein (blank nanoparticles).

$$EE(\%) = \frac{[lutein]_{total} - [lutein]_{non\ entrapped}}{[lutein]_{total}} x\ 100 \quad (3)$$

*5.2.5 Nanoparticle characterization*

In order to investigate possible chemical interactions between zein and lutein, Fourier Transform Infrared spectrometry (FTIR, Frontier Perkin Elmer) analyses were conducted with resolution of 1 $cm^{-1}$ from 4000 to 400 $cm^{-1}$. Differential scanning calorimetry (DSC, Perkin Elmer 4000) analyses were care out in order to investigate thermal behaviour of samples. The samples were accommodated in sealed aluminium pans with nitrogen flow (50 mL•min-1), in a range from 20 to 360 °C and heat flow of 10 °C.$min^{-1}$. X-ray diffraction analysis (XRD) was carried out to assess the impact of encapsulation technique on lutein crystalline conformation, proving its encapsulation in zein polymeric matrix. XRD analysis were conducted in an X-ray diffractometer (Bruker, D8 Advance) with scan range between 3° and 60° (2θ) at a rate of 5.9°.$min^{-1}$, using Cu Kα radiation generated at 40 KV and 35 mA. Average particles size (D) and

polydispersion index (PDI) were determined by Dynamic Light Scattering (DLS, Malvern Zetasizer - Nano Series) using backscattering detection (173°) in samples without previous dilution. PDI was calculated using the standard deviation (σ) of the sizes distribution curve by Equation 4.

$$PDI\ (-) = \frac{\sigma^2}{D^2} \quad (4)$$

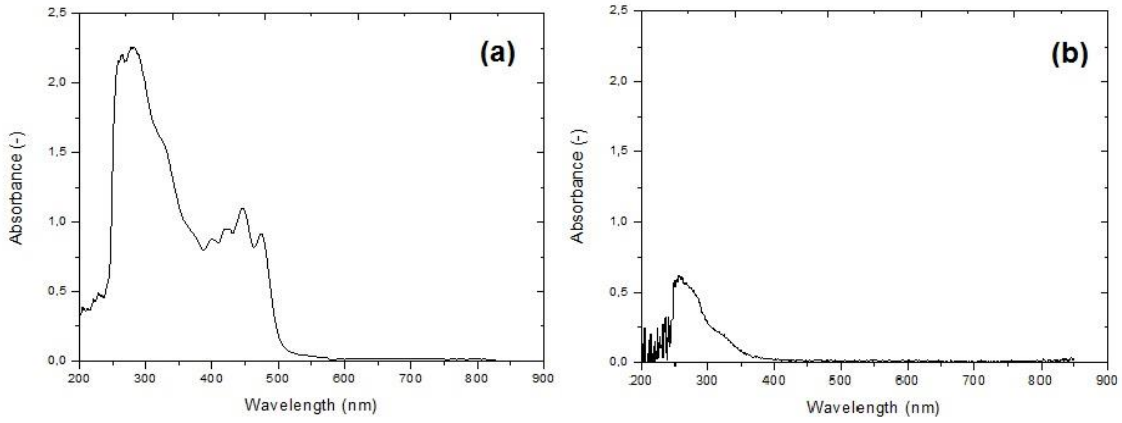
Morphological characterization of the nanoparticles was performed using Transmission Electron Microscopy (TEM; JEOL model JEM 2100, 200 kV). Diluted samples were dripped onto 300 mesh parlodium covered copper grids. Grids were dried at room temperature and stained with osmium tetroxide for 4 hours.

## 5.3 Results and Discussion

### *5.3.1 Analytical validation*

Fig. 3 presents the UV-Vis spectra of pure lutein and zein nanoparticles without lutein.

Figure 3 - UV-Vis spectra for: (a) pure lutein; (b) zein nanoparticles (without lutein).




The term specificity always refers to 0% interferences or 100% selectivity, in other words, no interferences are supposed to occur in the wavelength in which detection is carried out (ARAUJO, 2009). It is possible to

notice that no signal was detected at 446 nm in the blank nanoparticles spectrum meaning that this wavelength is specific to lutein. Thus, 446 nm was the wavelength selected for the calibration curve.

Lutein calibration curve found was “absorbance (-) = 0.008 + 4,344 * Lutein ($mg.mL^{-1}$)”. Linear and angular coefficients of calibration curve found were 0.008 ± 0.005 and 4.344 ± 0.043 $mL.mg^{-1}$, respectively. The adjusted correlation coefficient of the straight line was 0.999 and the standard error of estimate was 0.015 $mg.mL^{-1}$, which means that linearity can be assumed. Residuals presented normal distribution and there were no outliers. ANOVA shown that calculated F value (10244.845) was evidently higher than the tabulated F at a significance level of 1% (9.074).

The detection limit found was 1.454 $mg.L^{-1}$, indicating that this is the lowest concentration at which the analyte can be safely detected by the method. The quantification limit determined was 4.407 $mg.L^{-1}$. Dias, Camões and Oliveira (2008) have validated an High Performance Liquid Chromatography (HPLC) method to the quantitative determination of main carotenoids. The detection limit and quantification limit found by them was 0.0132 $mg.L^{-1}$ and 0.0399 $mg.L^{-1}$, respectively. Stinco and co-workers (2014) have validated an HPLC method to quantify carotenoids, tocopherols and chlorophylls. They obtained 0.006 $mg.L^{-1}$ and 0.020 $mg.L^{-1}$ as detection limit and quantification limit for lutein, respectively. Careri, Elviri and Mangia (1999) used the Reversed-Phase Liquid Chromatography – Electrospray Mass Spectrometry interfaced with TurboIonspray (LC – TurboISP – MS) to investigate β-carotene and xanthophylls. Although chromatographic methods presented lower values of quantification and detection limits, spectroscopic methods are worth developing since they are generally less time consuming, more environmental friendly and can be applied to an array of encapsulation techniques. Moreover, detection and quantification limits found here are quite enough to quantify lutein in nanoparticles since this compound is usually not added in lower concentration in encapsulation processes.

In Table 1 data used to calculate recovery rates and accuracy are presented.

Table 1 - Accuracy study for the analytical validation of the lutein determination method

| Concentration of added lutein ($mg \cdot L^{-1}$) | Sample (day) | Concentration of lutein found ($mg \cdot L^{-1}$) | Average of lutein concentrations found ($mg \cdot L^{-1}$)* | Recovery rate mean (%) (confidence interval 95%)* † |
|---|---|---|---|---|
| 240 | 1a | 233.7 | 233.5 RSD = 0.22% | 97.29 ± 0.22 [a] RSD = 0.22% |
| | 1b | 233.1 | | |
| | 1c | 233.1 | | |
| | 2a | 233.5 | | |
| | 2b | 233.3 | | |
| | 2c | 234.4 | | |
| 24 | 1a | 23.4 | 23.3 RSD = 1.02% | 97.03 ± 0.99 [a] RSD = 1.02% |
| | 1b | 23.1 | | |
| | 1c | 22.9 | | |
| | 2a | 23.4 | | |
| | 2b | 23.4 | | |
| | 2c | 23.6 | | |
| 6 | 1a | 4.0 | 4.1 RSD = 10.39% | 69.05 ± 7.18 [b] RSD = 10.39 % |
| | 1b | 3.6 | | |
| | 1c | 3.8 | | |
| | 2a | 4.5 | | |
| | 2b | 4.7 | | |
| | 2c | 4.3 | | |

RSD = Relative Standard Deviation.
* n = 6.
† Different letters in the same row indicate statistical differences ($p > 0.05$).

The effect of lutein concentration was statistically significant ($p < 0.05$) and the effect of “day x concentration” interaction was statistically significant only for the 6 $mg.L^{-1}$ concentration of lutein solution ($p > 0.05$). Recovery rate values varied from 97.29% to 69.05% and RSD from 0.22% and 10.39%. The statistical difference and high RSD values that occurred in the 6 $mg.L^{-1}$ lutein solution indicates that this method is less sensitive to low lutein concentration as expected. On the other hand, this UV-Vis method for lutein quantification showed increased sensibility for the highest and medium concentration.

Accuracy is the first role for an analytical method validation (GONZÁLEZ; HERRADOR; ASUERO, 2010). The acceptable recuperation rate depends on the objective of the analysis and, generally, it is within a range from 70% to 120%. This interval may change depending on the sample preparation and on the analytical procedure (SILVA-BUZANELLO et al., 2015).

Indyk and co-workers (2014) have validated an intra-laboratory method using liquid chromatography – ultraviolet technique to estimate lutein recovery

during infant formula manufacture. They achieved recovery rates of 95.5% - 109.5%, values that comply with the accepted criteria. An Ultra-high Performance Supercritical Fluid Chromatography for determination of carotenoids in dietary supplements was developed and validated by Li and colleagues (2015a). This method yielded lutein recovery rates of 100.6% to 109.5% and RSD of 3.2% to 6.0% and showed to be efficient to the determination of nine main carotenoids in dietary supplements.

Table 2 shows the results for repeatability (intra-day), intermediate precision (inter-day) and reproducibility (intra-laboratory).

Table 2 - Precision levels (repeatability, intermediate precision and reproducibility) for the analytical validation of the lutein determination method

| Lutein concentration | Analyst I | | | Analyst II | | | Reproducibility |
|---|---|---|---|---|---|---|---|
| | 1st day | 2nd day | Inter-day (n=6) | 1st day | 2nd day | Inter-day (n=6) | |
| (mg·L$^{-1}$) | RSD(%) | RSD(%) | RSD(%) | RSD(%) | RSD(%) | RSD(%) | RSD(%) |
| 240 | 0.66 | 0.18 | 0.42 | 0.45 | 0.09 | 0.27 | 0.34 |
| 24 | 1.11 | 2.34 | 1.73 | 2.00 | 1.31 | 1.65 | 1.69 |
| 6 | 3.71 | 3.92 | 3.82 | 4.44 | 3.00 | 3.72 | 3.77 |
| Lutein concentration | Laboratory I | | | Laboratory II | | | Reproducibility |
| | 1st day | 2nd day | Inter-day (n=6) | 1st day | 2nd day | Inter-day (n=6) | |
| (mg·L$^{-1}$) | RSD(%) | RSD(%) | RSD(%) | RSD(%) | RSD(%) | RSD(%) | RSD(%) |
| 240 | 0.66 | 0.18 | 0.42 | 0.55 | 0.35 | 0.45 | 0.43 |
| 24 | 1.11 | 2.34 | 1.73 | 1.02 | 1.54 | 1.28 | 1.50 |
| 6 | 3.71 | 3.92 | 3.82 | 4.68 | 4.3 | 4.47 | 4.14 |

RSD: Relative Standard Deviation (%)

Repeatability assays was carried out by the same analyst in same day (Analyst I, 1st day) and RSD% values remained between 0.66% and 3.71%. The maximum value of relative standard deviation obtained in intermediate precision assays (between analysts) was 3.77% and in reproducibility assays (between laboratories) was 4.14%.

Observing Table 2, it is possible to notice that RSD% increased for decreasing lutein concentration. Nevertheless, those results are satisfactory for quantification in nanoencapsulation systems, where the concentration of encapsulated active substances usually ranges from 1% to 10%.

Marinova and Ribarova (2007) validated an HPLC determination of carotenoids in Bulgarian berries. Their repeatibility assay showed relative

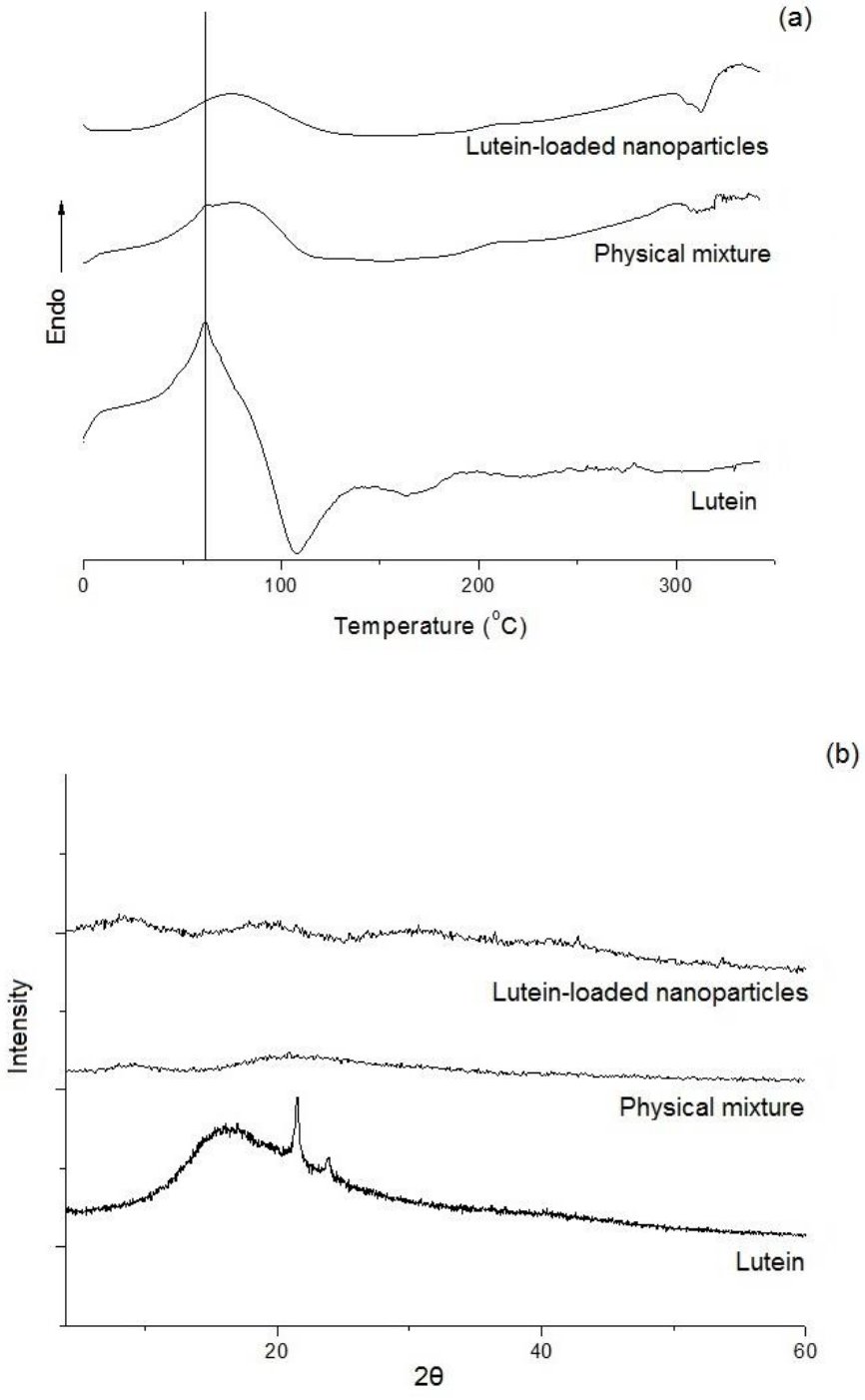
standard deviation of 2.5% for lutein quantification. An analytical validation for quantification of lutein and zeaxanthin in the aquous humor of single mouse eyes was done by Karlsen and coleguees (2003) using capillary HPLC. RSD values of precision was 2% for the highest lutein concentration and 13% for the lowest lutein concentration. Abrahamsson, Rodriguez-meizoso & Turner (2012) have developed and validated a method based on supercritical fluid chromatography for quantitative determination of carotenoids in extracts of *Scenedesmus* sp obtaining 6.4% RSD for intermediate-precision. Li and co-workers (2012a) validated an ultra-performance liquid chromatographic technique for separate geometric isomers of carotenoids from tomato cultivars. Reproducibility assay showed relative standard deviation of 7.3% for lutein and the authors considered their results highly reproducible.

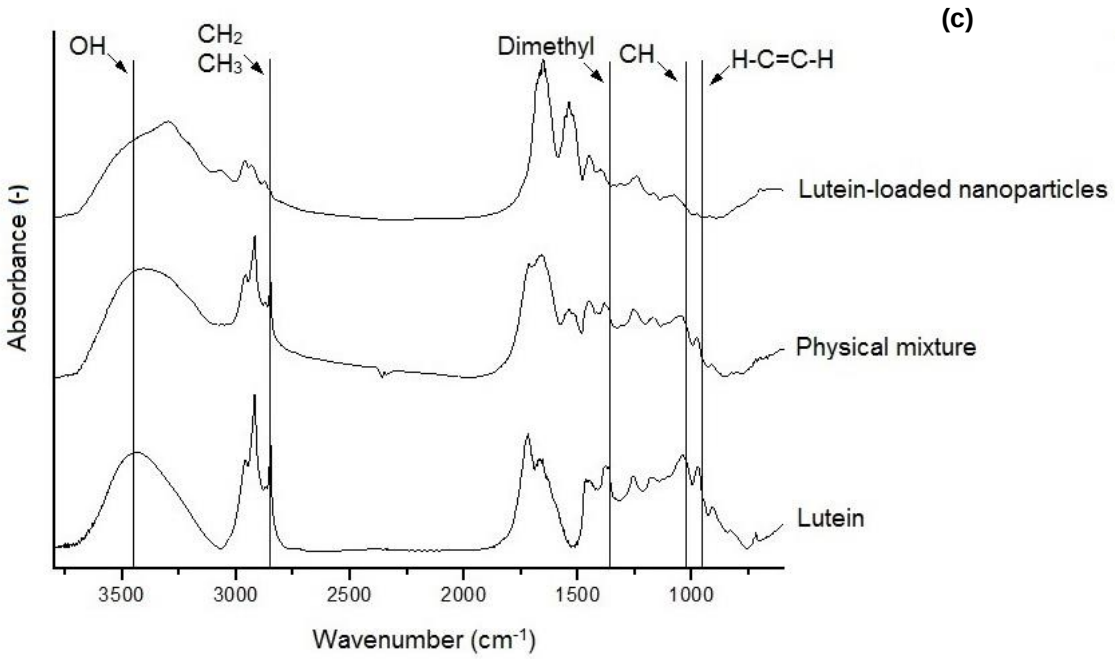
*5.3.2 Nanoparticles characterization*

Lutein concentration and entrapment efficiency in the nanoparticles were determined by the validated method. Values found were 87.9 ± 3.6 $mg.L^{-1}$ and 95.9 ± 1.4%, respectively. Most of researchers have been investigating lutein entrapment efficiency using HPLC technique. Hu and co-workers (2012) evaluated lutein entrapment efficiency in zein nanoparticles obtained by solution enhanced dispersion by supercritical fluids (SEDS) method. The entrapment efficiency was affected by the process variables, reaching values between 83.15% and 34.44%. Lacatusu and co-workers (2013) prepared lipid nanoparticles based on omega-3 fatty acids containing lutein. They obtained stable particles with 88.5% of entrapment efficiency. Arunkumar and colleagues (2015) encapsulated lutein in poly(lactic-co-glycolic acid)–polyethylene glycol nanocapsules. Authors observed that the concentration of the stabilizer have affected nanoparticles characterization and the maximum entrapment efficiency found was 88%. One may conclude that the entrapment efficiency presented here is in accordance with those presented in the literature. The high value of entrapment efficiency obtained suggests that both the chosen encapsulation technique (nanoprecipitation) and encapsulant substance (zein) are adequate to encapsulate lutein.

DSC thermograms, X-ray diffraction patterns and infrared spectra of lutein, lutein-PVP physical mixture and lutein-loaded nanoparticles are presented in Fig. 4 (a), (b) and (c), respectively.

Figure 4 - Characterization results of pure lutein, lutein-PVP physical mixture and lutein-loaded nanoparticles: (a) DSC thermograms, (b) XRD diffractograms and (c) FTIR spectra.






Zein glass transition occurs in 197.05 °C and 202.40 °C in lutein-loaded nanoparticles and physical mixture, respectively (Fig. 4.a). Those values are higher than those reported by literature for raw zein (PODARALLA; PERUMAL, 2012) probably due to crop variations. The broad endothermic peak around 75°C presented in the lutein-loaded nanoparticles and the physical mixture thermograms is characteristic of sodium caseinate (ZHANG; ZHONG, 2013). Lutein thermal analysis showed endothermic and exothermic peaks at 61.3°C ($\Delta H$ = 63.11 $J.g^{-1}$) and 107.2°C ($\Delta H$ = -79.64 $J.g^{-1}$), respectively, corresponding to degradation of its chemical structure (MIGUEL et al., 2008). Despite the low concentration of lutein in the nanoparticles, it was possible to observe the presence of a small peak around 62 °C in the physical mixture thermogram which corresponds to lutein. Also, such peak could not be detected in the lutein-loaded nanoparticles thermogram, suggesting that lutein was in amorphous state form distributed inside the nanoparticles, forming a homogenous solid mixture with zein.

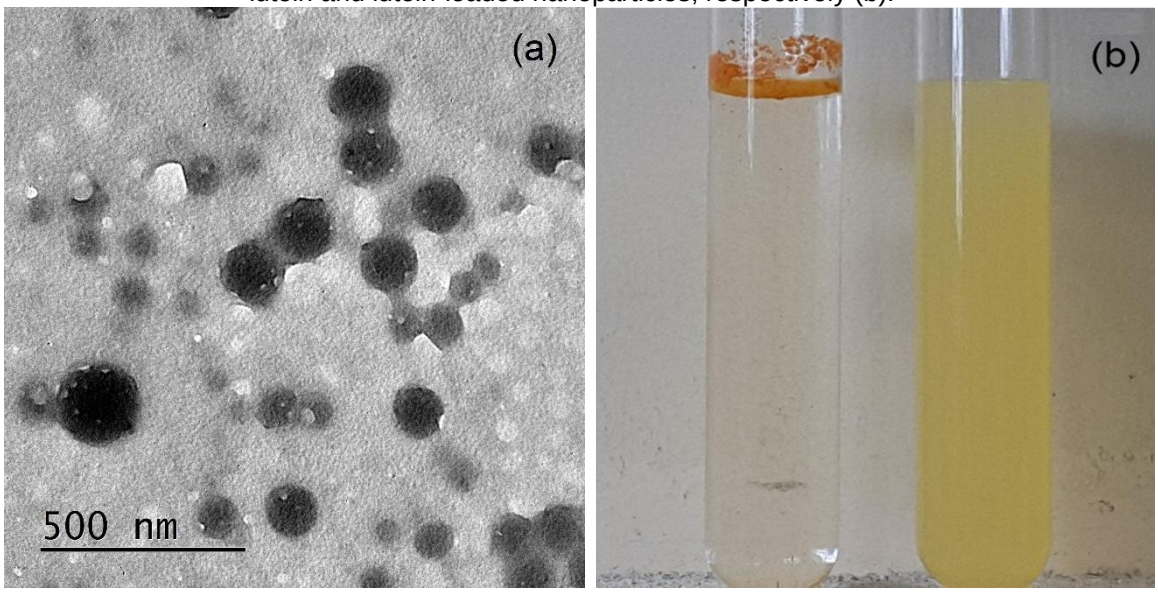
The strong peaks between 20° and 25° showed in Fig. 4.b indicated that pristine lutein presented crystalline nature. XRD difractogram of zein nanoparticles did not show crystalline peaks since zein is amorphous. The XRD diffractrogram of lutein-loaded nanoparticles also showed the absence of crystalline peaks, which means that the physical state of lutein was converted

from crystalline to amorphous due to encapsulation. The chemical interactions between lutein and polymer during nanoparticles preparation are able to convert the crystalline nature of lutein into an amorphous or disordered crystalline form (Arunkumar et al., 2015). Besides, the absence of crystalline peak in XRD diffractogram of lutein-loaded nanoparticles corroborated that there was not adsorbed lutein into nanoparticles surface but it was actually encapsulated into zein.

Infrared absorption spectrum of pristine lutein (Fig. 4.c) showed –OH peak at 3451 $cm^{-1}$, asymmetric and symmetric stretching vibrations of $CH_2$ and $CH_3$ at 2852 $cm^{-1}$, dimethyl group splitting at 1361 $cm^{-1}$ and trans conjugated alkene (–CH=CH-) out of plane deformation at 962 $cm^{-1}$ (NALAWADE; GAJJAR, 2015). Those characteristics peaks are in agreement with lutein chemical structure, which presents a long carbon chain with alternating single bounds and double bounds, methyl side groups and cyclic structures attached to hydroxyl groups (KIJLSTRA et al., 2012). All these peaks are also present in physical mixture along with others absorption peaks related to zein and sodium caseinate. Lutein characteristic groups could not be observed in the lutein-loaded nanoparticles spectrum, suggesting that lutein is located inside the nanoparticles. Moreover, absorption peaks presented in physical mixture are not present in lutein-loaded nanoparticles spectra, indicating that the chemical groups of lutein interacted with zein groups.

Fig. 5.a shows TEM image of lutein-loaded nanoparticles while Fig. 5.b presents and an image comparing pristine lutein dispersed in water with the lutein-loaded nanoparticles dispersion.

Figure 5 - TEM image of the zein nanoparticles containing lutein (a) and water dispersions: pure lutein and lutein-loaded nanoparticles, respectively (b).




Nanoparticles presented spherical shape and uniform size. This result is in agreement with DLS analysis, which found that particles presented average particles size of 242 ± 2 nm and polydispersion index of 0.380 ± 0.003. Moreover, it is possible to notice in Fig. 5 that pure lutein did not solubilize in water and remained embedded in the test tube. On the other hand, lutein-loaded nanoparticles were homogeneously dispersed in water confirming that encapsulation improved lutein water affinity. Arunkumar, Prashanth and Baskaran (2015) obtained lutein nanoencapsulated in low molecular weight chitosan. Transmission Electron Microscopy (TEM) images showed that particles were spherical in shape with particle sizes ranging from 80 to 600 nm. Liu and Wu (2010) also used TEM for lutein-loaded lipid nanoparticles with spherical shape and particle size of 134 nm.

## 5.4 Conclusions

A method for lutein quantification in zein nanoparticles using Ultraviolet-Visible technique was developed and validated. The validation procedure was carried out according to ICH guidelines evaluating specificity, linearity, accuracy and precision (repeatability, intermediate precision, and reproducibility). The method presented detection limit and limit of quantification of 1.454 $mg.L^{-1}$ and 4.407 $mg.L^{-1}$, respectively, which is appropriate to quantify lutein in typical

nanoencapsulation procedures. Relative standard deviation obtained in the assays indicate that this method is adequate to quantify lutein in nanoparticulated systems.

Furthermore, zein nanoparticles containing lutein was successful prepared using nanoprecipitation technique and the validated methodology was applied to determine lutein entrapment efficiency. Particles presented spherical shape with average particles size of 242 nm and 96.0% of entrapment efficiency.

# 6 NANOENCAPSULATION OF LUTEIN AND ITS EFFECT ON MICE'S DECLARATIVE MEMORY

## 6.1 Introduction

Dissolution in common solvent method is a viable strategy to encapsulate lipophilic bioactive and enhance theirs dissolution and bioavailability (FRIZON et al., 2013). However, it is worth investigate the actual improvement in the biological activity of lutein due to encapsulation because encapsulation procedures are often expensive and complex. The objective of this chapter was to encapsulate lutein in PVP nanoparticles and investigate its effect on *in vivo* mice's declarative memory.

These results were submitted as a manuscript to Material Science & Engineering C (ISSN 0928-4931).

## 6.2 Material and Methods

### *6.2.1 Material*

Lutein (90% purity, kindly gifted by Pincredit Bio-tech Co.), polyvinylpyrrolidone (PVP, 40,000 g.mol-1, Sigma-Aldrich), Tween 80 (Dinâmica) and ethanol (99.5%, Neon) were used in the nanoparticles preparation. Ethyl acetate (99.5%, Neon) and methanol (99.8%, Neon) were used as solvent in the nanoparticles solubility test. Olive oil was used for free lutein administration in mice by oral gavage.

### *6.2.2 Preparation of the lutein-loaded PVP nanoparticles*

Nanoparticles were prepared by the dissolution in common solvent method according to Karavas and co-workers (2006) with minor modifications as follows. Initially, PVP (mass depending on the formulation of each test) was dissolved in ethanol (7.5 mL) under magnetic stirring until the obtainment of translucent solutions. After that, lutein (15 mg) and Tween 80 (15 mg) were

added to this solution, remaining in mild agitation for five minutes. The obtained mixture was placed in ice bath and sonicated for 15 minutes under pulse condition of 30 seconds on and 10 seconds off (Fisher Scientific, 120W and 1/8' tip). Finally, the solvent was evaporated in a circulation oven at 40°C for 24 hours. Formulations were prepared with the following mass proportions of PVP:lutein: 0:1, 2:1, 4:1, 6:1, 8:1, 10:1, 12:1 and 14:1 (m:m).

*6.2.3 Nanoparticle characterization*

Morphological characterization of the nanoparticles was performed using Transmission Electron Microscopy (TEM; JEOL model JEM 2100, 200 kV). Diluted samples were dripped onto 300 mesh parlodium covered copper grids. Grids were dried at room temperature and stained with osmium tetroxide for 4 hours. Thermal properties of the nanoparticles were investigated by Differential Scanning Calorimetry (DSC, Perkin Elmer 4000). Samples were accommodated in sealed aluminium pans under nitrogen flow (50 $mL.min^{-1}$) and heated from 20 to 360°C at 20°$C.min^{-1}$. Fourier Transform Infrared (FTIR) spectra were acquired using a Frontier Perkin Elmer equipment in potassium bromide pellets, with resolution of 1 $cm^{-1}$ from 4000 to 400 $cm^{-1}$. X-ray diffraction analyses (XRD, Bruker, D8 Advance) were carried out from 3° to 60° (2θ) at 5.9°$.min^{-1}$, using Cu Kα radiation generated at 40 KV and 35 mA. For contact angle measures, nanoparticles were dispersed in ethanol and dripped onto a glass slide in duplicate. Ethanol was allowed to evaporate under dry atmosphere protected from light for 48 hours. Finally, plates were evaluated in a goniometer (Hamé-Hart) using drop of diiodomethane on the sample surface. A total of 10 measured for each glass slide carried carried out.

*6.2.4 Interaction between PVP and lutein*

The spectrophotometric method suggested by Karavas and co-workers (2006) was used to verify the magnitude of the interaction between the wall material (PVP) and the encapsulated compound (lutein). A lutein solution in ethanol was prepared (0.05 $mg.mL^{-1}$) followed by the gradually addition of PVP in order to obtain different mass proportions of PVP:lutein, ranging of 0:1 to

16:1 (m:m). Each obtained system was analysed by UV-Vis spectrophotometry (Ocean Optics, USB-650-UV-VIS Red Tide) at the maximum absorption peak of lutein (446 nm).

Equation 5 was used to correlate PVP proportion in the system with the interaction intensity (F) between PVP and lutein (KARAVAS et al., 2006), where $A_S$ and $A_0$ represent the absorbance value at 446 nm of the PVP:LUT system and pristine lutein, respectively.

$$F = \frac{A_S - A_0}{A_0} \quad (5)$$

*6.2.5 Phase-solubility studies of lutein-loaded PVP nanoparticles*

In order to verify the influence of PVP proportion in the solubility of the nanoparticles in water, formulations were evaluated according to the procedure described by Mura and co-workers (2003). Aliquots of each formulation were transferred to test tubes so that 5 mg of lutein was present in each tube. After that, 7 mL of water was added and the test tubes were stirred during 1 hour at 25°C. Solutions were filtered through Millipore membrane filter (0.45 µm) and 500 µL of the filtrate was lyophilized (Liotop, L101). Lastly, 2 mL solution of methanol:ethyl acetate 1:1 (v:v) was added to the lyophilized samples and were evaluated by UV-Vis spectrophotometry at 446 nm. The amount of lutein that passed through the filtration membrane (0.45 µm) was considered water soluble since particles below that average size tend to remain stable without noticeable precipitation.

*6.2.6 In vivo studies*

6.2.6.1 Animals

Adult male Swiss mice (90 – 60 days of age; n = 61) were used, weighing between 25 - 30 g each, from the Central Animal Laboratory at the State University of West Paraná. Animals were housed in polypropylene cages of 41 x 34 x 16 cm, under light controlled conditions (light/dark cycles of 12

hours) at (22 ± 2)°C, with food and water *ad libitum*. The animal use protocol followed the Official Ethical Guidelines of the Brazilian Government and the Ethics Committee on Animal Use (CEUA) of UNIOESTE and UTFPR. All experimental protocols were designed to minimize the number of animals used and their suffering, according to the project approved by the Ethics Committee, ordinance no. 2729/2014 – GRE.

Animals were divided into 6 groups and each group was fed by oral gavage during 14 days as following: saline solution (10 $mL.Kg^{-1}$), olive oil (10 $mL.Kg^{-1}$), pristine lutein (100 $mg.Kg^{-1}$) and lutein-loaded PVP nanoparticles with 8:1 (m:m) PVP:LUT mass proportion (1.5, 5.0 and 10.0 $mg.Kg^{-1}$).

6.2.6.2 Object recognition task

The object recognition task was carried out according to the protocol described by Bevins and Besheer (2006), which is composed of three distinct phases: habituation, training and testing, each one with duration of 10 minutes.

In habituation session, the wooden box has its floor divided into 12 equal areas and animals were free to explore the box. On this stage were counted the number of crossing and the frequency with which mice stood on their hind legs in the field (rearing).

In training session, mice were introduced to two identical objects and in testing session, one of the objects was replaced for a new one. The time spent to explore the familiar object (A) and the new object (B) was measured and the object discrimination index was calculated according to Equation 6.

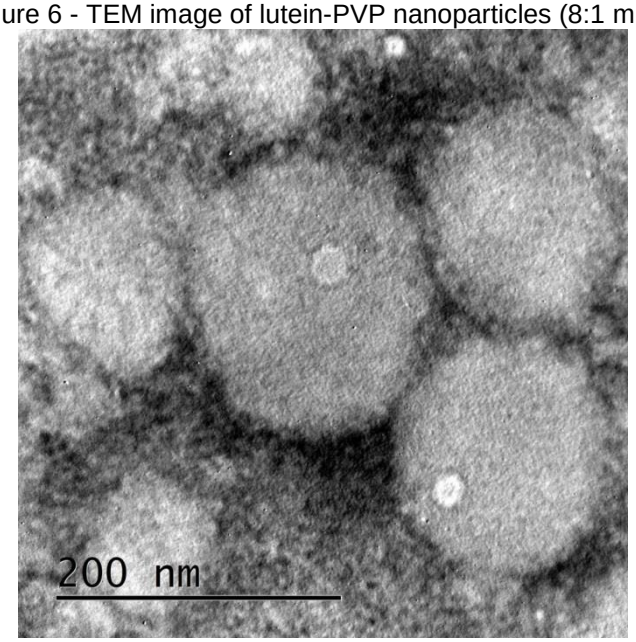
$$Object\ discrimination\ index\ (-) = \frac{(B - A)x100}{B + A} \quad (6)$$

## 6.3 Results and Discussion

### *6.3.1 Nanoparticles characterization*

Figure 6 shows TEM images of lutein-PVP nanoparticles in PVP:lutein mass ratio of 8:1 (m:m).

Figure 6 - TEM image of lutein-PVP nanoparticles (8:1 m:m).




Particles presented spherical shape and uniform size around 200 nm. As stated by Zhao and co-workers (2014), the mechanism of formation of lutein-loaded PVP nanoparticles is quite complex. Lutein is incorporated in PVP through hydrogen bonding between hydroxyl groups of lutein and carbonyl groups of PVP. This chemical bond is responsible for cross-links between these two compounds, favouring their precipitation of the alcoholic solution and the formation of the nanoparticles.

Figure 7 shows infrared spectra of pristine compounds (PVP and lutein), physical mixture (PVP, lutein and Tween 80) and lutein-loaded PVP nanoparticles in different mass ratios. Figure 8 shows DSC curves of pristine compounds (PVP and lutein), physical mixture (PVP, lutein and Tween 80) and lutein-loaded PVP nanoparticles in different mass ratios. Table 3 presents the obtained enthalpy values of endothermic peaks of lutein-loaded PVP nanoparticles. Figure 9 presents DRX patterns of pristine compounds (PVP and lutein) and lutein-loaded PVP nanoparticles in 8:1 mass ratio.

Figure 7 - FTIR spectra of PVP, lutein, physical mixture and lutein-PVP nanoparticles.

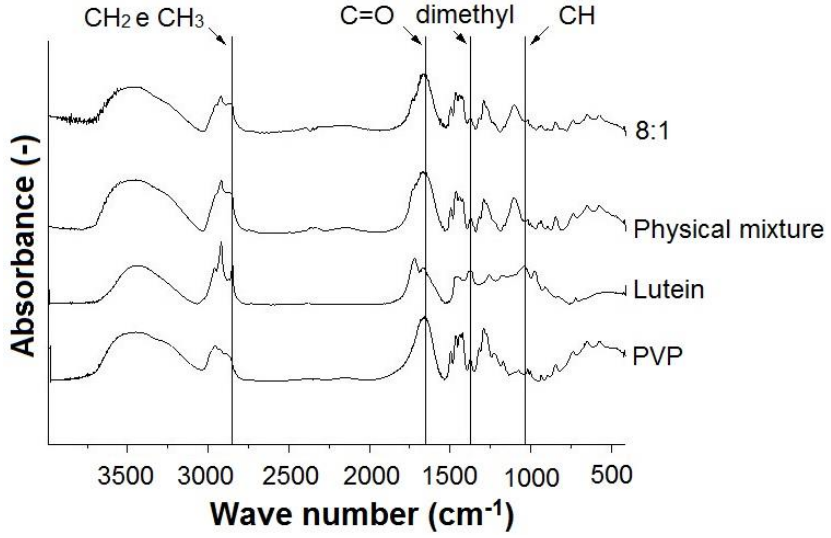



Figure 8 - DSC curves of PVP, lutein, physical mixture and lutein-PVP nanoparticles

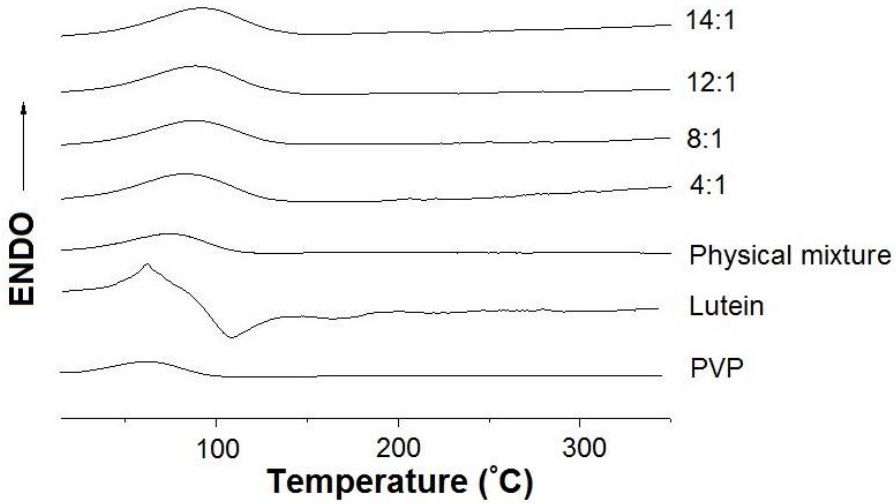
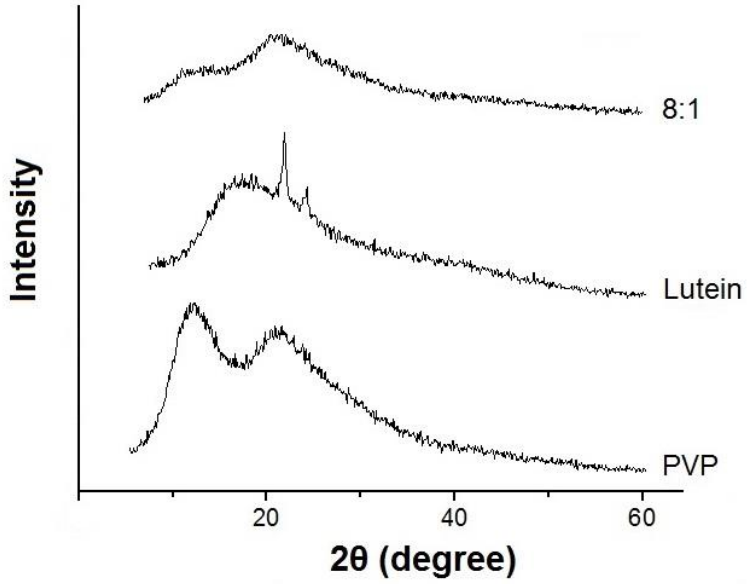



Table 3 - Temperature and enthalpy of the endothermic peak of the lutein-PVP nanoparticles.

| PVP:LUT (m:m) | Temperature (°C) | Enthalpy ($\Delta$H, J.g$^{-1}$) |
|---|---|---|
| 4:1 | 83.2 | 172.9 |
| 8:1 | 87.9 | 166.0 |
| 12:1 | 88.6 | 229.3 |
| 14:1 | 91.6 | 220.9 |

Figure 9 - XRD patterns of PVP, lutein and lutein-PVP nanoparticles.




Infrared absorption spectrum obtained for lutein is in accordance with that reported in literature (BOONNOUN et al., 2013). It is possible to identify the absorption bands related to CH (1036 $cm^{-1}$), dimethyl (1361 $cm^{-1}$), $CH_3$ and $CH_2$ (2852 $cm^{-1}$) groups, which was expected due to its chemical structure composed of a long carbonic chain, alternating single bounds and double bounds, also containing methyl side groups and cyclic structures attached to hydroxyl groups at each end of the chain (KIJLSTRA et al., 2012). Absorption band of the carbonyl group from PVP was detected at 1651 $cm^{-1}$. Those bands are evidenced in Figure 7, where it is possible to verify that they are also present in the physical mixture spectrum, indicating that there were no structural changes in components mixture. On the other hand, characteristic groups of lutein, most notably at 2852 $cm^{-1}$, was not detected in the nanoparticles spectrum suggesting that lutein was entrapped inside the nanoparticles.

DSC analyses allowed identification of a broad exothermic peak for lutein around 107.2 $^{\circ}C$ ($\Delta H = 79.6$ $J.g^{-1}$), which is often related to the degradation of its chemical structure (MIGUEL et al., 2008). This peak could not be seen in the physical mixture probably due to the low lutein concentration.

PVP is an amorphous polymer and, for this reason, it did not present melting peak (JIANG; MO; YU, 2012). Therefore, the endothermic peak at 67.3°C must be attributed to the evaporation of residual moisture (PARADKAR et al., 2004). The higher the PVP proportion in nanoparticles, the higher the

enthalpy of water evaporation peak of adsorbed water in nanoparticles, which is an indicative of the magnitude of water and PVP interaction. Increasing lutein concentration led to less intense water-PVP binding suggesting that less carbonyl groups were available to interact with water molecules and that PVP and lutein were forming hydrogen bonds inside the nanoparticles.

XRD pattern of pristine lutein showed two strong peaks between 20° and 25°, which confirms the lutein crystalline nature. XRD pattern of pure PVP did not show crystalline peaks since PVP is an amorphous polymer. Crystalline peaks were absent in the XRD pattern of lutein-PVP nanoparticles which means that the physical state of lutein was converted from crystalline to amorphous due to encapsulation and the chemical interactions between lutein and PVP during nanoparticles preparation (ARUNKUMAR et al., 2015).

### *6.3.2 Interaction between PVP and lutein and water solubility of the lutein-loaded PVP nanoparticles*

Figure 10 shows the correlation between PVP concentration in nanoparticles and contact angle of PVP and PVP-lutein films with diiodomethane. Absorption spectroscopy in ultraviolet and visible region enabled the quantitative investigation of interaction by hydrogen bonds established between PVP and lutein. Figure 11 presents the absorption spectrum of pure compounds and PVP:LUT system (14:1, m:m) and the interaction intensity between PVP and lutein in ethanol, under different proportions.

Figure 10 - Contact angle of lutein-loaded PVP nanoparticles (4:1, 8:1, 12:1 and 14:1) and pure PVP in diiodomethane. *Significant difference ($p<0.05$) compared to pure PVP.

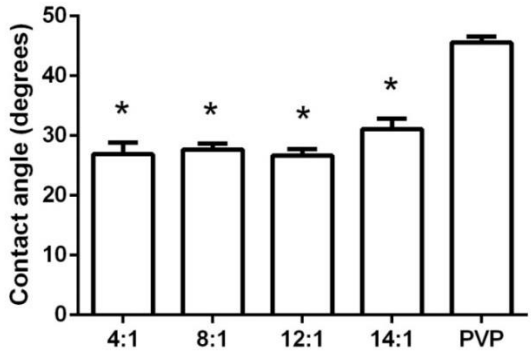
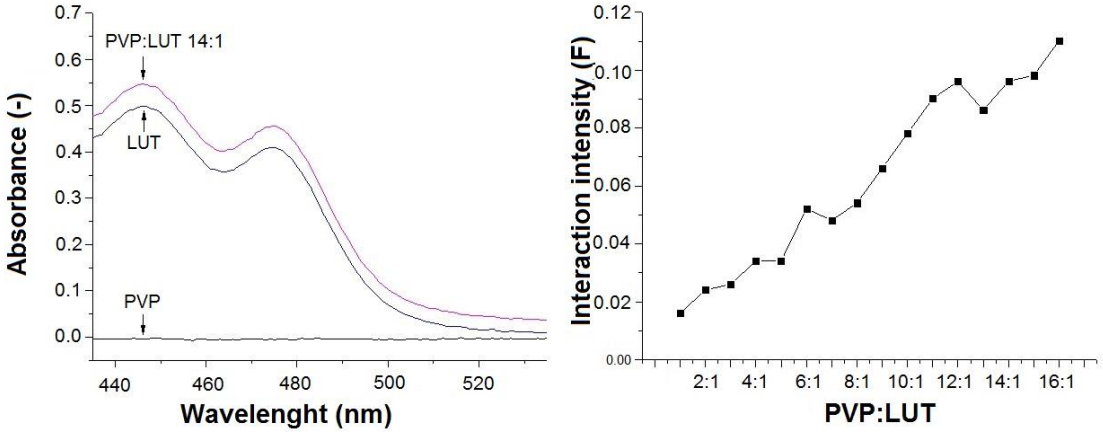


Figure 11 - UV-Vis absorption spectra of pure compounds and PVP:LUT system (14:1 m:m) and the interaction intensity between PVP and lutein.

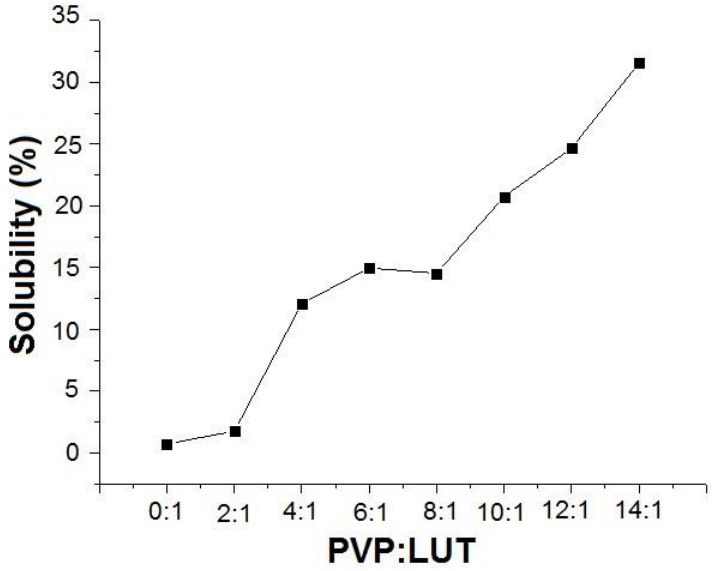



Contact angle measurements were used by Karavas and co-workers (2006) to study the nature of the interactions between PVP and the hydrophobic compound felodipine. However, to determine how lutein and PVP interact each other, a hydrophobic liquid was chosen (diiodomethane) because PVP is prone to rapidly dissolve in water which may difficult the correct interpretation of the results. As one may see in Figure 10, pure PVP presents a high contact angle value confirming the hydrophilic nature of PVP. Even small additions of lutein decreased the contact angle, showing that the interaction between PVP and lutein generated a more hydrophobic compound than pure PVP.

The interaction assays were carried out in the same solvent used in the encapsulation procedure. For this reason, it can be assumed that the obtained results on this assay correspond to interaction intensity during nanoparticles formation (KARAVAS et al., 2006). Besides, PVP did not absorb energy in the visible region of the spectrum which was also demonstrated in Figure 11. PVP:LUT system showed higher absorbance than pure lutein, known as hyperchromic effect, at the same lutein concentration, as can be seen in Figure 11a. The increase in absorbance observed in PVP:LUT system is attributed to the presence of chemical interactions between the polymer and lutein, by the formation of hydrogen bonds between hydroxyl (lutein) and carbonyl (PVP) groups. The increasing interaction intensity between PVP and lutein with increasing PVP concentration was in accordance with the changes found in moisture loss temperatures and enthalpies presented in the DSC curves (Figure 8) further corroborating the mechanism of the nanoparticles formation.

Figure 12 shows the influence of different PVP:LUT mass ratios on the solubility of the lutein present in the nanoparticles.

Figure 12 - Water solubility of the lutein-loaded PVP nanoparticles produced with different PVP and lutein mass proportions.




It was possible to notice a considerable increase in the water solubility of lutein with increasing PVP:LUT proportions, which is in agreement with the contact angle and UV-Vis results. In this assay, (0.72 ± 0.19) % of the pristine lutein added was dissolved in water while lutein-loaded PVP nanoparticles reached values around (31.55 ± 1.72) %. In this sense, encapsulation technique was able to increase by more than 43 times lutein water solubility, which may be attributed to the formation of soluble complexes between bioactive compound (lutein, hydrophobic) and the polymer (PVP, hydrophilic) (ZHAO et al., 2014). Karavas and co-workers (2006) studied felodipine-PVP nanodispersions. In this case, PVP was able to increase 30 times the water solubility of felodipine by the formation of soluble complexes established by hydrogen bonds. Sethia and Squillante (2004) evaluated the influence of PVP in the water solubility of carbamazepine, one of the major drugs used in the treatment of epilepsy. Authors have studied nanodispersions with different concentration of PVP and different molar mass (1 to 20%) and the results showed that carbamazepine solubility increased linearly reaching 12 times more soluble than pristine carbamazepine.

Frizon and co-workers (2013) prepared PVP nanodispersion containing loratadine, an anti-alergenic drug. Authors observed that PVP was able to increase loratadine water solubility probably by improving drug wettability, increasing surface area due to small particle size and converting the drug from crystalline to amorphous state. Manju and Sreenivasan (2011) synthetized curcumin-PVP conjugates by an esterification step. Those conjugates were able to improve curcumin solubility as well and could potentially improve its medicinal application. Mura, Falcci and Bettinetti (2001) evaluated PVP influence in naproxen complexes with hydroxypropyl-β-cyclodextrin (HPβCD). The results showed a synergetic effect between PVP and HPβCD, increasing the aqueous solubility of naproxen (120 times of the pure drug), contributing with about 65% increase in apparent stability constant of the naproxen-HPβCD complex in presence of only 0.1% (w/v) PVP.

*6.3.3 In vivo studies*

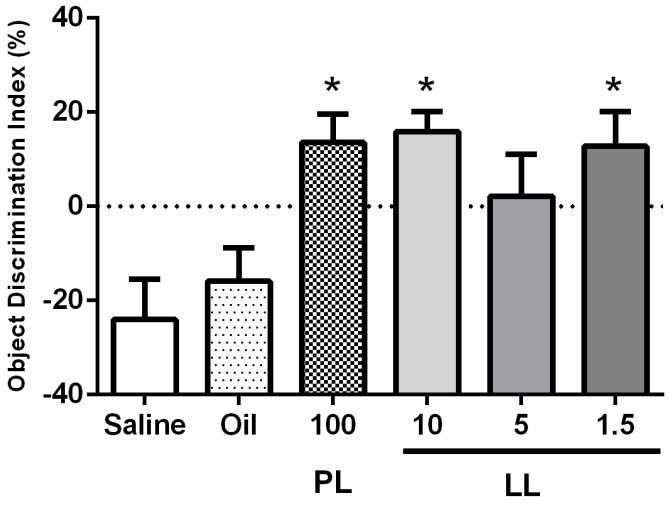
Table 4 presents the effect of free lutein (pristine) and lutein-loaded PVP nanoparticles (8:1, m:m) on the exploratory behavior of mice during habituation session. Figure 13 shows the effect of oral administration of free lutein and lutein-loaded PVP nanoparticles on the discrimination index in the test session of the object recognition task.

Table 4 - Effect of free and encapsulated lutein in exploratory behavior of animals during habituation session

| Groups | Crossing | Rearing | N |
|---|---|---|---|
| **Saline** | 153.9 ± 8.8 | 44.5 ± 4.2 | 10 |
| **Olive oil** | 140.4 ± 12.4 | 47.4 ± 4.3 | 10 |
| **Free Lutein 100 mg.Kg$^{-1}$** | 156.0 ± 7.3 | 56.9 ± 3.8 | 11 |
| **Lutein-PVP nanoparticles 10 mg.Kg$^{-1}$** | 167.1 ± 7.3 | 53.1 ± 3.6 | 11 |
| **Lutein-PVP nanoparticles 5 mg.Kg$^{-1}$** | 141.0 ± 12.7 | 47.8 ± 5.7 | 10 |
| **Lutein-PVP nanoparticles 1.5 mg.Kg$^{-1}$** | 148.6 ± 8.6 | 46.6 ± 2.2 | 9 |
| **Statistical analysis** | $F_{(5.55)}$ = 1.131<br>p > 0.05 | $F_{(5.55)}$ = 1.306<br>p > 0.05 | |

Results expressed as mean ± standard error of the mean; N: number of animals in each group.

Figure 13 - Object discrimination index (PL: free lutein (pristine); LL: lutein-loaded PVP nanoparticles). *Significant difference (p<0.05) compared to the vehicle.




Spontaneous locomotor activity using open field tasks were measure to rule out the possible interference of change in the parameters of learning and memory. As one may see in Table 4, there was no significant difference ($p>0.05$) among treatments. This result indicates that lutein administration in free and encapsulated form did not cause motor impairment of animals, therefore, did not influence its locomotion during object recognition task.

Statistical analysis showed that there were statistical differences in the discrimination index among the mice groups evaluated ($F_{(5,55)} = 5.70$; $p < 0.05$). Furthermore, Bonferroni's test identified that groups in which free lutein (100 $mg.Kg^{-1}$) and lutein-loaded PVP nanoparticles was administrated at doses of 10 and 1.5 $mg.Kg^{-1}$ differed statistically of the groups which was administrated the vehicle (olive oil and saline). This indicated that the administration of free lutein at 100 $mg.Kg^{-1}$ and lutein-loaded PVP nanoparticles 10 and 1.5 $mg.Kg^{-1}$ significantly increased mice's object recognition index, revealing that lutein administration improved memory in the object recognition task.

It may be noted that similar effect was achieved by free lutein at 100 $mg.Kg^{-1}$ and lutein-loaded PVP nanoparticles in lower doses (1.5 and 10 $mg.Kg^{-1}$). This may be related to the increase in the lutein water solubility caused by its encapsulation. As depicted above, PVP and lutein form a soluble complex and particularly in this case, the proportion of PVP and lutein used for encapsulation procedure (8:1, m:m) increased 20 times its solubility. The increased solubility of lutein probably culminated in increasing its bioavailability,

which provides greater absorption by the body and enhancing its biological effect.

Currently, evidence suggests that lutein promotes neuroprotective effects *in vivo*. A study conducted by Li and co-workers (2012b) showed that lutein presents protective action on mice's brain after the induction of ischemic stroke. This effect isattributed to the anti-apoptotic, antioxidant and anti-inflammatory properties of lutein. Authors claimed that lutein post-stroke regimen may be a potential treatment for stroke patients. Miyake and co-workers (2014) evaluated lutein action in mice's neuronal cell line. In this study, the treatment of neuronal cell lines with lutein reduced free radicals intracellular levels and induced phase II antioxidative enzyme expression.

## 6.4 Conclusions

Lutein-loaded PVP nanoparticles were prepared by the nanodispersion method, presenting spherical morphology. Encapsulation technique enabled the increase of lutein water solubility in more than 43 times due to formation of a soluble complex through hydrogen bonds between PVP carbonyl group and lutein hydroxyl group. Such interaction between PVP and lutein was further detected by other characterization techniques such as DSC, DRX, UV-Vis, goniometry and FTIR.

*In vivo* studies demonstrated that the oral administration of free lutein (100 $mg.Kg^{-1}$) and lutein-loaded PVP nanoparticles (1.5 and 10 $mg.Kg^{-1}$) during 14 days significantly increased object discrimination index in the object recognition task. Furthermore, similar effect ($p< 0.05$) was achieved by free lutein and lutein-loaded PVP nanoparticles in doses 66 times lower, demonstrating that encapsulation was able to potentiate the activity of lutein in this case. In this sense, lutein may be considered as an effective pharmacological alternative in memory deficits treatment, however, further studies are needed to evaluate the mechanism of action involved in this effect.

## 7 CONCLUSÕES GERAIS

Um método para a quantificação de luteína em nanopartículas de zeína utilizando a técnica de ultravioleta-visível foi desenvolvida e validada. O procedimento de validação seguiu o guia do ICH, avaliando especificidade, linearidade, acurácia e precisão (repetibilidade, precisão intermediária e reprodutibilidade). O método apresentou limite de detecção e limite de quantificação de 1.454 $mg.L^{-1}$ e 4.407 $mg.L^{-1}$, respectivamente, sendo apropriado para quantificar a luteína em procedimentos típicos de nanoencapsulação. O desvio padrão relativo obtido nas análises indicam que esse método é adequado para quantificar luteína em sistemas nanoparticulados.

Com a finalidade de aplicar o método validado, nanopartículas de zeína contendo luteína foram preparadas utilizando a técnica de nanoprecipitação. As partículas apresentaram formato esférico com diâmetro médio de 242 nm e 96,0% de eficiência de encapsulação.

Em outra parte do trabalho, nanopartículas de PVP contendo luteína em diferentes proporções foram preparadas através do método de nanodispersão. As partículas apresentaram formato esférico e tamanho em torno de 200 nm, conforme confirmado por Microscopia Eletrônica de Transmissão. A técnica de encapsulação possibilitou o aumento da solubilidade da luteína em mais de 43 vezes devido à formação de um complexo solúvel formado por ligações de hidrogênio entre a carbonila do PVP com a hidroxila da luteína. Estudos *in vivo* demonstraram que a administração de 100 $mg.Kg^{-1}$ de luteína livre e 10 e 1,5 $mg.Kg^{-1}$ de luteína encapsulada durante 14 dias aumentou significativamente ($p<0,05$) o índice de discriminação de objetos de camundongos na tarefa de reconhecimento de objetos. Ainda, efeito semelhante ao alcançado pela luteína livre pôde ser atingido utilizando luteína encapsulada em doses 66 vezes menores.

Deste modo, a encapsulação da luteína foi capaz de potencializar o efeito deste carotenoide sobre o cérebro, fornecendo uma alternativa farmacêutica eficaz para o tratamento de déficits de memórias.

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