UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ENGENHARIA DE ALIMENTOS

WANESSA COSTA SILVA FARIA

"GREEN COFFEE FRUIT EXTRACT: MICROCAPSULES DEVELOPMENT BY SPRAY DRYING TECHNIQUE, ANTIOXIDANT EFFECTIVENESS, AND STABILITY AND SAFETY ASSESSMENT FOR FOOD APPLICATIONS"

"EXTRATO DO FRUTO DO CAFÉ VERDE: DESENVOLVIMENTO DE MICROCÁPSULAS POR SPRAY DRYING, EFICÁCIA ANTIOXIDANTE E AVALIAÇÃO DA ESTABILIDADE E SEGURANÇA PARA USO EM ALIMENTOS"

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Tese apresentada à Faculdade de Engenharia de Alimentos da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutora em Ciência de Alimentos.

Supervisor/Orientadora: NEURA BRAGAGNOLO

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A Ata de Defesa, assinada pelos membros da Comissão Examinadora, encontra-se no processo de vida acadêmica do aluno.

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RESUMO

O café, em sua composição fitoquímica, apresenta riqueza em compostos bioativos, em especial os ácidos clorogênicos (ACGs), aos quais são atribuídos inúmeros benefícios à saúde incluindo a atividade antioxidante, antiobesogênica, anti-hipertensiva e anticancerígena. Como o café em seu estágio imaturo contêm maior concentração destes compostos, tanto a indústria alimentícia quanto a farmacêutica têm apresentado interesse nos grãos do café verde para a produção de extrato ou mesmo isolamento dos ACGs para diversos usos. Entretanto, muitos estudos demonstram que o extrato bruto possui maior eficácia frente aos ACGs isolados visto que outras classes de metabólitos secundários presentes no café podem agir sinergicamente otimizando a atividade funcional dos ACGs. No presente estudo, foram otimizados os processos de extração por percolação dos compostos bioativos do fruto do café verde bem como o desenvolvimento e a padronização de micropartículas contendo extrato do fruto do café verde concentrado por meio de planejamento experimental, pela técnica de secagem por aspersão. Inicialmente foi aplicado um delineamento composto central rotacional (DCCR) 2^2, na qual a concentração de etanol no solvente (%, m/m) e a proporção de material vegetal para solvente (m/m) foram estudadas visando otimizar o rendimento do processo e o conteúdo de ACGs no extrato. Em seguida, outro DCCR 2^2, avaliou a influência de diferentes proporções de goma arábica (GA) e maltodextrina (MD) como agentes encapsulantes do extrato do fruto do café verde (EFCV) e diferentes proporções da dispersão contendo agentes encapsulantes e EFCV em relação à eficiência do encapsulamento, rendimento do processo, retenção de ácidos clorogênicos, estabilidade dos compostos bioativos e da capacidade redutora das micropartículas obtidas. A melhor condição obtida para a extração de ACGs baseado no planejamento e na superfície de resposta, utilizando ácido 5-*O*-cafeoilquinico (5-CQA) como marcador foi: concentração de etanol entre 65 a 80% (m/m) e proporção de matéria-prima para solvente entre 0,8:10 – 1:10 (m/m). Já no processo de microencapsulação, o maior rendimento foi obtido quando 80% da GA foram substituídas pela MD e utilizando a proporção de 1:3,5 (m/m) de extrato concentrado em relação a dispersão contendo material de parede. A capacidade antioxidante do extrato e micropartículas otimizadas foram analisadas em óleo de girassol, sendo que as micropartículas demonstraram serem mais eficazes em atrasar o processo de oxidação do óleo comparado ao EFCV livre e ao antioxidante sintético butil hidroxitolueno (BHT). Estudos preditivos de toxicidade *in silico* dos compostos majoritários identificados no EFCV demonstraram que a cafeína apresenta uma menor LD_{50} frente ao 5-CQA, ácido cafeico, entre outros compostos analisados. Além disso, estudo de toxicidade aguda determinou a DL_{50} do EFCV livre na dose de 5000 mg/kg p.c. em camundongos machos e fêmeas, no entanto, a DL_{50} do extrato microencapsulado não causou a morte de nenhum camundongo na mesma dosagem. Nos ensaios de toxicidade subaguda conduzidos em ratos machos, a administração oral de 1000 mg/kg p.c. do extrato microencapsulado durante 30 dias não causou nenhum efeito adverso nos animais. Deste modo, a NOAEL (no-observed-effect-adverse level) atribuída ao EFCV microencapsulado é de 1000 mg/kg p.c./dia, sendo a dose equivalente calculada para o consumo humano de 189 mg/kg p.c./dia.

ABSTRACT

The phytochemical composition of coffee shows a large amount of bioactives compounds, specially the chlorogenic acids (ACGs), which are attributed a number of health benefits comprising antioxidant capacity, antihypertensive, anti-obesogenic, anticancer effect, among others. Coffee in the first stage of the growth presents higher concentrations of these compounds. Food, cosmetic and pharmaceutical industries have presented interest on the green coffee beans to produce extracts, or even to isolate the chlorogenic acids and other compounds to apply in different products. However, various studies show that crude extract is more effective against isolated ACGs, since other class of phytochemicals provided by coffee can act synergistically optimizing the functional activity of ACGs. In this study, two different designe of experiment were applied as to optimize the bioactives extraction process by percolation using green coffee fruit as the matrix, well as for the development and standardization of microencapsulation process of the fluid green coffee fruit extract (GCFE) by spray drying technique. At first step, was applied a central composite rotational design (CCRD) 2^2, where ethanol concentration (%, w/w) and solid to solvent proportion (w/w) were assessed in order to optimize the yield process and the content of ACGs in the extract. Subsequently, other CCRD 2^2 was applied to evaluate the influence of different amount of gum Arabic (GA) and maltodextrin (MD) (%, w/w), and rate of concentrated extract to carrier agent dispersion (w/w) on encapsulation efficiency, process yield, 5-*O*-caffeilquinic acid (5-CQA) retention, stability of bioactives compounds and reducing capacity of the microparticles obtained. The best condition found to ACGs extraction based on response surface, using 5-CQA as chemical marker, were ethanol concentration range of 65 – 80 % (w/w) and solid to solvent proportion in the range of 0.8:10 – 1:10 (w/w). The antioxidant capacity of the non-encapsulated and encapsulated GCFE obtained in the best condition of optimization process were analyzed in sunflower oil, in which encapsulated GCFE showed be more effective in delay the oil oxidation than non-encapsulated GCFE and synthetic antioxidant butyl hydroxytoluene (BHT). The predictive *in silico* study of the major compounds identified in GCFE achieved a smaller lethal dose 50 (LD_{50}) to caffeine compared to 5-CQA, caffeic acid, among other analyzed. Besides, *in vivo* acute toxicity study found the oral single dose of non-encapsulated at 5000 mg/kg bw as LD_{50}, both in female and male mice. However, the LD_{50} of encapsulated GCFE was not found in this study since the dose of 5000 mg/kg bw of this product did not cause any death. On the subacute study carried out in male rats, the 30-day oral administration of encapsulated GCFE at highest dosage selected (1000 mg/kg bw) did not cause any adverse effect in rats. In this way, the no-observed-adverse-effect level (NOAEL) attributed to encapsulated GCFE is 1000 mg/kg bw/day. And the calculated equivalent dose to human consume is 189 mg/kg bw/ day.

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CAPÍTULO I

INTRODUÇÃO GERAL E OBJETIVOS

1. INTRODUÇÃO GERAL

O café provém de uma árvore do gênero *Coffea* e dentre as várias espécies cultivadas no mundo as mais comercializadas são *Coffea arabica* e *Coffea canephora*, conhecidas respectivamente por arábica e robusta (MONTEIRO; TRUGO, 2005). Mundialmente consumido, este grão é uma das *commodities* mais comercializadas no mundo. De acordo com a *International Coffee Organization* (2017), o volume de exportação do grão foi de aproximadamente 7,34 milhões de toneladas em 2016/2017, estimado ao valor de 20,1 bilhões de dólares. Dentre os seus produtos (café verde ou cru, torrado e moído e café solúvel), o grão verde ou cru é o que apresenta maior volume de exportação (BRASIL, 2016).

O grão cru apresenta em sua composição química riqueza em polifenóis, sendo os ácidos clorogênicos (ACGs) a principal classe, os quais são formados pela esterificação de ácidos *trans*-cinâmicos (ácidos cafeico, ferúlico e *p*-cumárico) com o ácido quínico (SOGA; OTA; SHIMOTOYODOME, 2013). Estudos científicos que avaliaram a concentração de ACGs nos frutos imaturos do café verificaram que o fruto, contendo a casca e a polpa, mantêm maior proporção destes compostos bioativos quando comparados aos grãos verdes despolpados (NOBRE et al., 2011). Além disso, quanto mais verde estão os frutos (estágios I e II), maior é proporção de ACGs de acordo com KOSHIRO et al. (2007).

Evidências científicas têm demonstrado que os compostos fenólicos provenientes do extrato do grão do café cru ou verde (ECV), principalmente os ACGs, exibem inúmeras propriedades biológicas, incluindo efeitos antibacteriano, antioxidante, atividade anticarcinogênica e termogênica (KONO et al., 1997; SHIMODA; SEKI; AITANI, 2006; VINSON; BURNHAM; NAGENDRAN, 2012). Além de modular o metabolismo da glicose e dos lipídeos, tanto em indivíduos saudáveis como em portadores de distúrbios metabólicos (ZHANG et al., 2011; MUBARAK et al., 2013). Suas atividades podem também ser exploradas em matrizes *ex-vivo*, tal como alimentos processados e óleos vegetais ricos em ácidos graxos insaturados, onde podem atuar como um antioxidante natural (BUDRYN et al., 2015; BUDRYN; ZACZYNSKA; ORACZ, 2016).

Entretanto, para que estes efeitos sejam reprodutíveis, é de suma importância que o extrato obtido do fruto do café verde nos estágios I e II DE maturação (EFCV) apresente constância de qualidade. E, para que este extrato tenha importância comercial, é mister que este produto tenha ainda reprodutibilidade no seu rendimento. Todavia, tais aspectos estão sujeitos

a diversas variáveis que constituem dificuldades a serem superadas. No caso de produtos naturais obtidos de plantas, as condições de cultivo, época de coleta, métodos extrativos empregados, secagem e as condições de armazenagem (CASTRO et al., 2004) são as variáveis que devem ser consideradas. Devido a esses vários fatores, torna-se necessária a utilização de marcadores químicos para o acompanhamento da qualidade e para a padronização desses produtos (CHAVES, 2005). Marcador químico consiste na substância ou conjunto de substâncias que estejam presentes em uma espécie vegetal e que preferencialmente sejam responsáveis pela atividade biológica da espécie (BRASIL, 2012) que no caso do EFCV são os ACGs expressos em ácido 5-*O*-cafeoilquínico (5-ACQ).

Para a obtenção de um extrato padronizado rico em compostos bioativos, em especial os ACGs, frutos inteiros do café imaturo (chumbão) foram utilizados como materia-prima. E, para que o mesmo possa ser utilizado tanto na indústria alimentícia quanto pela indústria farmacêutica, torna-se necessário definir parâmetros físicos e físico-químicos desejáveis, como concentração de marcadores químicos, distribuição granulométrica e morfologia das partículas (CALIXTO, 2000). Além disso, a eficácia desses compostos também depende da sua preservação, sendo este o maior obstáculo na aplicação destes bioativos no desenvolvimento de nutracêuticos e aditivos naturais, já que são suscetíveis à degradação durante o processamento quando submetido a diferentes condições de temperatura, luz e oxigênio (KHASAEI et al., 2014).

Para melhorar a estabilidade de ingredientes tais como antioxidantes, bioativos, corantes, vitaminas e outros produtos, tanto a indústria farmacêutica como a indústria de alimentos tem lançando mão de diferentes métodos de microencapsulação que consistem na produção de minúsculas partículas de ingredientes sólidos, líquidos ou gasosos empacotados em micropartículas diminutas onde seus compostos são liberados em taxas controladas sob a influência de determinados estímulos (SOBRINHO; FARIAS, 2012).

No setor alimentício, a padronização e a microencapsulação de produtos bioativos visando garantir sua estabilidade físico-química e, consequentemente, estabilidade funcional é de suma importância, tendo em vista que alimentos para fins de saúde já são tendência fundamentada neste setor. Além disso, a substituição de aditivos alimentares sintéticos por aditivos naturais, tem ganhado atenção da indústria alimentícia devido a demanda de público com interesse em uma alimentação mais equilibrada e saudável, livre de ingredientes sintéticos (TAGHVAEI; JAFARI, 2015). Frente a isso, nestes últimos anos houve um aumento do número de produtos alimentícios com alegações de saúde, assim como alegações de troca de ingredientes sintéticos por naturais, nas gôndolas de supermercados e lojas especializadas

(MORAES; COLLA, 2006; BRASIL FOOD TRENDS 2020, 2010). A Secretaria de Vigilância Sanitária (ANVISA) por meio da Resolução nº 18, de 30 de abril de 1999 estabelece que toda alegação funcional ou de saúde deva ser comprovada por evidências científicas tais como: composição química com caracterização molecular; ensaios bioquímicos; ensaios nutricionais e ou fisiológicos e ou toxicológicos em animais de experimentação; estudos epidemiológicos; ensaios clínicos; evidências abrangentes da literatura científica e comprovação de uso tradicional, observado na população, sem associação de danos à saúde (BRASIL, 1999a).

Além da estabilidade e da funcionalidade, outro requisito importante no desenvolvimento de um produto é a segurança no seu consumo. A Resolução nº 02, de 07 de janeiro de 2002, da ANVISA, que regulamenta a incorporação de substâncias bioativas em alimentos, estabelece que a segurança destas substâncias deve ser averiguada através de ensaios de toxicidade *in vivo*, conforme o estabelecido pela Resolução nº 17, de 30 de abril de 1999 (BRASIL, 1997; BRASIL, 1999b; BRASIL 2002). O estudo de toxicidade irá estabelecer o Nível de Efeito Adverso não Observado (NOAEL) e a Ingestão Diária Aceitável (IDA), que é a dosagem que pode ser ingerida diariamente sem comprometer à saúde do indivíduo. A ANVISA através da Portaria SVS/MS nº 540/1997, estabelece que, substâncias direcionadas para serem utilizadas como aditivos alimentares ou coadjuvantes de tecnologia devem ser alvos de estudo que comprovem a segurança para que seja estabelecida a IDA.

Ensaios preliminares de toxicidade, utilizando ferramentas computacionais *in silico* têm sido amplamente empregados para prever possíveis efeitos adversos, dose letal, ou seja, a dose que causa a morte de 50% dos indivíduos tratados (DL_{50}), o potencial mutagênico e carcinogênico, e etc., de novas substâncias ou mesmo de extratos vegetais, através de análises de predição química utilizando os marcadores químicos selecionados ou, mais comumente, substâncias majoritárias contidas no extrato (SALGUEIRO et al., 2016; AMMAR, 2017). Entretanto, métodos computacionais tem o papel de complementar estudo de toxicidade *in vitro* e *in vivo*, dado que, ensaios *in vivo* são determinantes para a certificação da NOAEL e da IDA de um novo ingrediente (RAIES; BAJIC, 2016).

Diante do apresentado, o desenvolvimento e a otimização do processo de extração de bioativos dos frutos imaturos do café e da microencapsulação dos extratos obtidos pelo método de secagem por atomização ou *spray drying*, além da aplicação de testes de estabilidade e eficácia antioxidante em produtos alimentícios, e ainda, estudos da segurança *in silico* e *in vivo* dos compostos majoritários presentes no extrato utilizando o extrato não encapsulado assim como as micropartículas obtidas, foram realizados devido a inexistência de um produto similar

e ainda que contemplem as exigências da legislação nacional de modo que possa ser aplicado no processo de produção de alimentos como um ingrediente ou substância bioativa.

2. OBJETIVOS

2.1 OBJETIVO GERAL

Desenvolver métodos para o processo de extração e de microencapsulação do extrato hidroetanólico padronizado em ácido clorogênico a partir de frutos do café conilon (*Coffea canephora*) nos estágios I e II de maturação, constituídos pela casca, polpa, mucilagem, pergaminho e semente; avaliar a estabilidade dos compostos majoritários encontrados no extrato não encapsulado e no extrato microencapsulado bem como a estabilidade da capacidade redutora *in vitro* durante testes acelerados de vida de prateleira. Além de conduzir ensaios de segurança *in vivo* a fim de definir a dose letal aguda e a dosagem na qual não são observados efeitos adversos nos animais (NOAEL).

2.2 OBJETIVOS ESPECÍFICOS

- Processar e caracterizar fisico-quimicamente frutos de café verde, *Coffea canephora*, clone 83, provenientes da Empresa de Pesquisa Agropecuária de Minas Gerais (EPAMIG) unidade Urezm, localizada no município de Viçosa, Minas Gerais;
- Obter o extrato fluido dos frutos de café verde com maior concentração de ácidos clorogênicos e/ou maior rendimento através de delineamento estatístico experimental composto central rotacional;
- Identificar e quantificar o ácido clorogênico 5-CQA nos extratos fluídos por cromatografia líquida de alta eficiência acoplado a detectores de arranjo de diodos e espectrometria de massas;
- Otimizar o processo de microencapsulação do EFCV variando as proporções de materiais de parede (maltodextrina e goma arábica) e proporções de extrato através de delineamento estatístico experimental do tipo composto central rotacional;
- Identificar e quantificar os componentes químicos majoritários, incluído o marcador químico 5-CQA, no extrato microencapsulado e extrato não encapsulado;

- Analisar as micropartículas em termos de eficiência da microencapsulação, retenção dos ácidos clorogênicos expressos em 5-CQA, rendimento do processo, morfologia, umidade total e atividade de água;
- Avaliar a estabilidade dos ácidos clorogênicos expressos em 5-CQA, cafeína, trigonelina e do ácido cafeico no extrato não encapsulado e nas micropartículas obtidas durante testes acelerados de vida de prateleira determinando o conteúdo desses compostos por HPLC e a capacidade redutora *in vitro;*
- Desenvolver extrato não encapsulado e micropartículas através dos métodos de extração e microencapsulação otimizados para o café Robusta a fim de comparar seu potencial como aditivo antioxidante em relação aos produtos do café Arábica em matriz alimentícia;
- Verificar o potencial antioxidante de extratos não encapsulados e micropartículas contendo extrato de café arábica ou café Robusta em óleo de girassol por meio de ensaios que determinam a formação de produtos da oxidação primária e secundária, durante estudo de vida de prateleira do óleo suplementado ou não com esses produtos;
- Avaliar o potencial de perigo dos compostos majoritários quantificados no extrato de café Robusta através de analises preditivas *in silico*, a fim de nortear posterior estudo de toxicidade *in vivo;*
- Analisar a toxicidade aguda tanto do extrato não encapsulado quanto das micropartículas em camundongos machos e fêmeas, visando observar os efeitos adversos agudos, além de delimitar a dose letal 50 (DL_{50});
- Prever o possível efeito estimulante do extrato não encapsulado em camundongos machos por meio de ensaio comportamental de locomoção;
- Analisar a toxicidade subaguda do produto do café com menor potencial tóxico, determinado na análise de toxicidade aguda, em ratos machos, com o objetivo de determinar o NOAEL.

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CAPÍTULO II

REVISÃO BIBLIOGRÁFICA

1. Padronização de extratos vegetais

O processo de padronização de um extrato vegetal consiste em ajustar as condições ótimas para obtenção de um produto com qualidade e rendimento reprodutíveis que apresente um marcador utilizado como referência no controle da qualidade da matéria-prima vegetal (BRASIL, 2014).

O marcador é definido como substância ou classe de substâncias (ex.: alcaloides, flavonoides, ácidos graxos, etc.) que, preferencialmente, apresente correlação com o efeito terapêutico. Segundo a Resolução nº 26 de 13 de maio de 2014, o marcador pode ser do tipo ativo, quando relacionado com a atividade terapêutica do fitocomplexo, ou analítico, quando não demonstrada, até o momento, sua relação com a atividade terapêutica do fitocomplexo (BRASIL, 2014).

Diversos produtos padronizados são encontrados no mercado brasileiro, principalmente medicamentos fitoterápicos como: *Ilex paraguariensis* (erva-mate), *Camellia sinensis* (chá verde) e *Paullinia cupana* (guaraná), ambos padronizados em metilxantinas; *Brosimun gaudichaudii* (mamacadela), padronizado em psoraleno e bergapteno, *Panax ginseng* (Ginseng), padronizado em ginsenosídeos; *Mikania glomerata* (guaco), padronizado em cumarinas; *Hypericum perforatum* (hipérico), padronizado em hipericina); *Passiflora incarnata* L. (maracujá), padronizado em vitexina (BARATA, 2005; CARVALHO et al., 2008).

De acordo com a Farmacopeia brasileira (2010) pode-se obter três formas farmacêuticas de extrato padronizado: extrato fluído, extrato mole e extrato seco, que se diferenciam entre si, principalmente, pela quantidade de resíduo seco no produto final. O extrato seco é obtido pela evaporação parcial do solvente utilizado na preparação devendo apresentar, no mínimo, 95% de resíduo seco (p/p), calculado como percentagem de massa. Os extratos secos padronizados podem ter o teor de seus constituintes ajustados pela adição de materiais inertes adequados ou ainda pela adição de extratos secos com o mesmo fármaco utilizado na preparação. Os extratos moles possuem consistência pastosa devido à evaporação parcial do solvente utilizado na preparação e apresentam, no mínimo, 70% do resíduo seco (p/p). Já os extratos fluidos são preparações líquidas nas quais, uma parte do extrato corresponde a uma parte, em massa, da droga seca utilizada na sua preparação. Esses podem ser padronizados, em termos de concentração do solvente, teor de constituinte ou resíduo seco. Os extratos fluidos e moles podem ser obtidos por percolação ou por dissolução de extratos secos ou moles utilizando como

solvente unicamente o etanol, água ou etanol/água na proporção adequada (FARMACOPEIA BRASILEIRA, 2010).

Os ensaios de pureza especificados para extratos fluidos compreendem a determinação da densidade, etanol, 2-propanol, metanol e do resíduo seco (FARMACOPEIA BRASILEIRA, 2010).

Para a padronização de extratos vegetais é de suma importância a aplicação dos métodos analíticos de validação que possam ser aplicados em diferentes etapas do processo, tal como no processo extrativo e/ou no processo de secagem. Além disso, devem ser analisadas as propriedades físico-químicas nessas diferentes etapas de obtenção do extrato a fim de definir parâmetros físicos e químicos desejáveis, como concentração de marcadores químicos, propriedades organolépticas, distribuição granulométrica e morfologia das partículas em se tratando de extrato seco (CALIXTO, 2000).

2. Secagem por atomização

O método de secagem por *spray drying* é um método aplicado desde a metade do século 18, época na qual foi patenteada a primeira operação de secagem de ovos por Larmont em 1965 (MASTERS, 1991). Porém, o início da sua utilização em processos industriais se deu a partir da década de 20, quando foi utilizada pela primeira vez, em escala industrial para a produção de leite em pó que, até hoje, continua sendo a aplicação industrial mais importante deste método (CAL; SOLLOHUB, 2010).

O equipamento *spray dryer* ou secador por atomização admite a alimentação somente de material em estado fluído (solução, suspensão, emulsão ou pasta) o qual é transformado em um produto particulado seco através da nebulização do líquido e a exposição da névoa formada a uma substância gasosa aquecida (Figura 1) (MASTERS, 1991). De acordo com Bimbenet (1978), quando um alimento é colocado em uma corrente de ar com baixa umidade relativa e elevada temperatura (150 – 300ºC), é formada, espontaneamente, uma diferença de temperatura e pressão parcial de água entre o alimento e o ar, resultando uma transferência de energia na forma de calor do ar para o produto e uma transferência de água do produto para o ar.

A popularização da técnica de *spray drying* ocorreu durante a segunda guerra mundial, sendo intensivamente aplicada para a redução do peso e volume de alimentos e também para otimizar a conservação dos mesmos durante o transporte e a estocagem (MASTERS, 2002). A

partir de então, seu uso disseminou-se pela indústria de processos, sendo hoje, aplicada especialmente para secagem em larga escala de produtos das linhas alimentícia e farmacêutica.

Na indústria alimentícia a técnica de secagem por atomização tem sido amplamente aplicada para aumentar a estabilidade de extratos obtidos a partir de frutas, devido a perecibilidade destes alimentos na forma *in natura*. No entanto, a alta concentração de carboidratos tal como monossacarídeos (glicose e frutose), dissacarídeo (sacarose) e polissacarídeos e a complexidade da composição química faz com que os pós obtidos por spray drying tenham alta higroscopicidade e fiquem propensos a problemas de viscosidade e fluxo (KRISHNAIAH; NITHYANANDAM; SARBATLY, 2014). O grão de café verde é constituído por 60 g/100g de carboidratos em base seca incluindo polissacarídeos solúveis e insolúveis (celulose, arabinolagactana e galactomanana), oligossacarídeos (estaquiose e rafinose), dissacarídeos (sacarose) e monossacarídeos (glicose, galactose, arabinose, frutose, manose, manitol, xilose e ribose) (LUDWIG; CLIFFORD; LEAN et al., 2014). O pó obtido da secagem por atomização do grão de café verde pode ter características indesejáveis tal como pegajosidade, comportamento este atribuído a alta concentração de açúcares de baixo peso molecular e ácidos orgânicos que apresentam baixa temperatura de transição vítrea, e que podem, inclusive, interferir no funcionamento do *spray dryer* devido à possibilidade de entupimento do bocal e aderência a parede da câmara do aparelho. Entretanto, de acordo com Ludwig et al. (2014) este problema pode ser sanado com o uso de excipientes que, além de reduzirem a higroscopicidade do extrato seco obtido, ainda auxiliam na proteção contra a degradação térmica dos componentes bioativos presentes no extrato. Maltodextrina (MD) e goma arábica (GA) são geralmente utilizados como excipientes em extratos de frutas obtidos por atomização em *spray dryer*, devido a solubilidade e baixa viscosidade destes polissacarídeos, características importantes para o processo de secagem por atomização. Além disso, estes excipientes, que apresentam alto peso molecular, são úteis no aumento da temperatura de transição vítrea desses produtos (MOREIRA et al., 2009).

As etapas do processo de secagem por atomização estão intimamente relacionadas a qualidade do produto obtido. Assim, a otimização de parâmetros como temperatura de entrada e saída, concentração e tipos de adjuvantes tecnológicos, bem como, o teor de resíduo seco do extrato fluído são fatores indispensáveis para obtenção de extratos secos com melhores características físico-químicas e aumento do rendimento (VASCONCELOS et al., 2005).

Figura 1. Formação de partícula pelo método de secagem por atomização (OLIVEIRA; PETROVICK, 2010).

De acordo com Soares (2002), a temperatura do ar de entrada é um dos fatores determinantes, visto que o aumento na temperatura do material de entrada facilita o processo de secagem por reduzir a tensão superficial e a viscosidade o que facilita a formação de gotículas. Deste modo, a temperatura de entrada deve estar acima do ponto de ebulição do solvente utilizado. Entretanto, produtos alimentícios e biofarmacêuticos são geralmente termossensíveis o que torna necessário, por vezes, o uso de adjuvantes com atividade tensoativa que auxiliam na redução da tensão superficial do material e propiciam a formação de gotículas com menores tamanhos, além de aumento na velocidade de atomização (SOARES, 2002).

A distribuição e o tamanho da partícula dependem do controle sobre a dimensão média das gotículas formada pelo processo de atomização, diante disso, a escolha do bico aspersor é fundamental para obtenção de pós com granulometria uniforme o que permite controlar as propriedades do pó como planejado.

Existem no mercado diferentes bicos aspersores, classificados em: pneumáticos, de pressão, de disco giratório (OLIVEIRA; PETROVICK, 2010) e de bico ultrassônico

(KRISHNAIAH et al., 2014), sendo este último o que fornece gotículas mais uniformes e, portanto, partículas do pó produzido com tamanhos e distribuição relativamente homogêneas.

3. Métodos de obtenção de micropartículas

O método de microencapsulação é definido como um processo em que minúsculas partículas ou gotículas, denominadas de núcleo, são envolvidas por um revestimento ou incorporadas em uma matriz homogênea ou heterogênea, resultando em pequenas esferas com diversas utilidades. Sendo que o material de parede pode ser composto de um ou mais ingredientes que fornecerá uma camada única ou dupla à cobertura (GHARSALLAOUI et al., 2007).

Segundo Krishnaiah et al. (2014), técnicas de microencapsulação podem ser divididas em três classes: processos químicos como inclusão molecular ou polimerização interfacial, processos físico-químicos como coacervação, emulsificação e encapsulação em lipossomas e processos físicos tal como *spray drying*, *spray chilling/coolling*, co-cristalização, extrusão ou revestimento por leito fluidizado.

Diferentes tipos de micropartículas e microesferas são produzidos a partir de uma ampla variedade de materiais de parede (monómeros e/ou polímeros), pelas diferentes técnicas de microencapsulação utilizadas e, ainda, pelas propriedades físico-químicas do núcleo, caracterizadas por: esferas simples rodeadas por revestimento de espessura uniforme; partículas contendo um núcleo de forma irregular; várias partículas de núcleo incorporadas numa matriz contínua de material de parede; vários núcleos distintos dentro de uma mesma cápsula e microcápsula com multicamadas de material de parede (figura 2) (GHARSALLAOUI et al., 2007).

Tais processos têm fundamental importância na indústria de alimentos e vem sendo amplamente utilizada com a finalidade de controlar ou preservar compostos de aromas e pigmentos, bem como, ingredientes bioativos, vitaminas, óleos essenciais, fitosteróis, ácidos graxos poli-insaturados, carotenoides e compostos antioxidantes diversos. A microencapsulação pode ser utilizada também para estabilizar moléculas, mascarar sabores e odores desagradáveis, além de permitir liberação controlada de compostos ativos (SANGUANSRI; AUGUSTIN, 2007; LIU et al., 2012).

Shahidi & Han (1993) propuseram seis razões para aplicação do processo de microencapsulação na indústria de alimentos: para reduzir a reatividade do núcleo com fatores

ambientais, diminuir a taxa de transferência do material do núcleo para o ambiente exterior, para facilitar a manipulação, para controlar a liberação do material do núcleo, para mascarar o sabor do núcleo e, por fim, diluir o material do núcleo quando ele deve ser utilizado em mínimas quantidades.

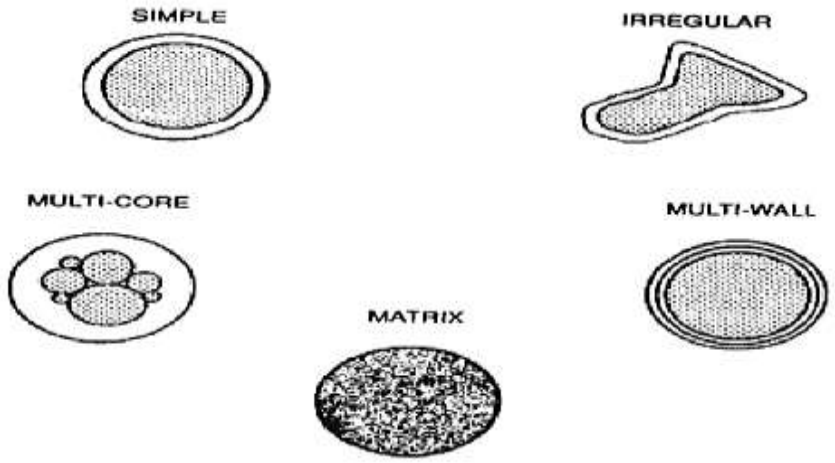


Figura 2. Morfologia de diferentes tipos de micropartículas (GHARSALLAOUI et al., 2007).

A seleção do método é dependente da aplicação que será dada à microcápsula, do tamanho desejado, do mecanismo de liberação e das propriedades físico-químicas, tanto do material ativo, quanto do agente encapsulante, que podem ser de natureza lipídica, proteica, glicídica ou ainda formado de materiais inorgânicos como sulfato de cálcio e silicatos (FAVARO-TRINDADE; PINHO; ROCHA, 2008). Os mecanismos de liberação dos materiais ativos microencapsulados irão variar de acordo com a natureza destes agentes, tais mecanismos geralmente ocorrem devido a variações de temperatura e de pH, solubilidade do meio, biodegradação, difusão, ruptura mecânica, permeabilidade seletiva e gradiente de concentração existente em relação ao meio de liberação. Cabe ressaltar que a espessura da cobertura da microcápsula pode ser modificada de forma que a estabilidade e a permeabilidade sejam alteradas (SOBRINHO; FARIAS, 2012).

4. Microencapsulação por *spray drying*

O processo de secagem por atomização é amplamente aplicado no processamento de diversos materiais para obtenção de grânulos, pós, microesferas e microcápsulas, cujo o objetivo principal é a retirada da água, responsável por propiciar um meio reacional adequado para reações químicas e enzimáticas, fenômenos físicos e proliferação microbiana (OLIVEIRA; PETROVICK, 2010).

De acordo com Mujumdar (2006), o processo de *spray drying* conduz a remoção térmica de substâncias voláteis (umidade) para obtenção de um produto sólido de diferentes morfologias que será definida pelo tipo de bico aspersor utilizado, agentes carreadores e outros.

Quando um sólido é submetido à secagem térmica, dois processos ocorrem simultaneamente:

1- Transferência de energia (principalmente na forma de calor) do ambiente para evaporação da umidade superficial.
2- Transferência da umidade interna para a superfície do sólido e subsequente evaporação devido ao processo 1.

A secagem industrial se difere em tipo e em planejamento de processo, dependendo do principal método de calor empregado. Na maior parte dos casos o calor é transferido para a superfície do sólido úmido, e em seguida, para o interior. No entanto, em dielétrica, rádio frequência, ou liofilização por micro-ondas, a energia é fornecida para gerar calor no sólido internamente que irá fluir para a superfície externa (MUJUMDAR, 2006),

Vale ressaltar também que conteúdo de água em um sólido pode estar livre ou ligada e que para a remoção da água ligada existem dois métodos: evaporação e vaporização. A evaporação ocorre quando a pressão de vapor da água na superfície do sólido é igual à pressão atmosférica. Neste aspecto, métodos industriais, tal como o sistema de secagem por spray-dryer, que combinam pressão, ar quente e atomização podem ser eficientes, tanto na secagem da água ligada quanto na água livre contida em um fluído (MUJUMDAR, 2006).

A remoção de água por spray drying tanto para a produção de extratos secos quanto para a obtenção de microcápsulas, tem sido adotada devido a sua capacidade de gerar um produto com especificações de qualidade precisas em operações contínuas, o que é requerido para obtenção de um produto padronizado, de maior estabilidade físico-química e microbiológica, alta concentração e estabilidade das substâncias ativas contidas no vegetal (OLIVEIRA; PETROVICK, 2010; KRISHNAIAH; NITHYANANDAM; SARBATLY, 2014). Além disso, auxilia na obtenção de um produto com propriedades funcionais específicas, evita o risco da

degradação química ou biológica das substâncias de interesse e, finalmente, reduz o custo de transporte e armazenamento (BAKRY et al., 2016).

De acordo com Bakry et al. (2016) a microencapsulação por spray dryer é uma tecnologia de baixo custo comumente utilizada em escala industrial, que tem como uma vantagem atrativa a produção de microcápsulas em uma operação contínua e relativamente simples quando comparada a outras técnicas de microencapsulação. Além disso, o processo oferece uma substancial flexibilidade na variação da matriz encapsulante resultando em partículas de boa qualidade.

A secagem por aspersão envolve a atomização de emulsões, suspensões coloidais ou dispersões em uma câmara a uma temperatura relativamente alta, que leva a uma rápida evaporação da água e, consequentemente, a formação rápida e quase simultânea de uma crosta e aprisionamento do núcleo (TONON et al., 2011).

O processo de microencapsulação por spray dryer envolve quatros estágios, que consistem de (i) preparação da dispersão ou emulsão, (ii) homogeneização da dispersão ou emulsão, (iii) atomização da emulsão ou da dispersão obtida, e (iv) desidratação da partícula atomizada. Em suma, no primeiro estágio o material de parede é dissolvido em água destilada ou outro veículo que solubilize o material encapsulado com auxílio de um agitador (SILVA et al., 2013; BAKRY et al., 2016). As soluções são mantidas por uma noite (± 12 horas) em temperatura ambiente ou refrigerada para assegurar a completa saturação do polímero, o material do núcleo é adicionado à solução com ou sem a adição de um agente emulsificante. No processo de secagem por *spray dryer*, o diâmetro inicial das gotículas ou partículas na mistura são na faixa de 0,1 a 100 µm (SILVA et al., 2013; BAKRY et al., 2016).

A viscosidade da emulsão ou solução final obtida e a distribuição do tamanho das partículas apresentam efeitos significantes no processo de microencapsulação por *spray-drying*. A viscosidade deve estar baixa o suficiente para prevenir a inclusão de ar nas partículas e as partículas devem estar em baixas dimensões. Ao observar estes fatores, se torna possível evitar a formação de partículas largas e alongadas que afetam adversamente a taxa de secagem. Além disso, a alta viscosidade interfere diretamente no processo de atomização, pois o material terá maior propensão em se aderir na parede da câmara de secagem devido ao baixo escoamento (SILVA et al., 2013; ANANDHARAMAKRISHNAN; PADMA, 2015; BAKRY et al., 2016).

5. Maltodextrina e goma arábica no processo de microencapsulação

Os critérios utilizados na seleção do material de parede para obtenção de microcápsulas ou microesferas são baseados nas propriedades físico-químicas como solubilidade, peso molecular, carga iônica, propriedades de transição vítrea, ponto de fusão, cristalinidade, difusibilidade, formação de filme e propriedades emulsificantes. Além disso, os fatores morfológicos resultantes no processo de microencapsulação que incluem distribuição do tamanho das partículas, forma, uniformidade de cobertura, teor de umidade, estabilidade físico-química e higroscopicidade (DRUSCH; DIEKMANN, 2015).

As maltodextrinas (MDs) são biopolímeros originados da hidrólise parcial do amido, estas são classificadas pelo seu grau de hidrólise, expresso em dextrose equivalente (DE), que é a percentagem de açúcares redutores calculados como glicose em relação ao peso seco do amido, possuindo uma média de 5 a 10 unidades de glicose/molécula. A definição de MD segundo a *Food and Drug Administration* (FDA), é a de um sacarídeo, não adocicado e nutritivo, que consiste de unidades de D-glicose unidas por ligações α (1-4) e que apresenta DE menor que 20 (FDA, 2015).

Em geral, as MDs são carboidratos de baixa densidade, totalmente solúveis em água. Na indústria de alimentos, as MDs podem ser utilizadas como agente espessante, para auxiliar a secagem por atomização, substituto de gordura, formador de filmes, no controle do congelamento, para prevenir cristalizações e complemento nutricional.

Maltodextrinas (MD) com DE entre 10 e 20 são as mais indicadas na utilização para o encapsulamento de aromas e outras substâncias voláteis, pois protegem substância da oxidação (CHRISTELLE; ELISABETH, 2013). MD com menor DE (5 a 7) apresentam um maior peso molecular (>4000g/mol) o qual confere uma maior estabilidade durante o processo de secagem devido a sua maior temperatura de transição vítrea, fazendo com que a secagem por atomização possa ser realizada de forma segura, abaixo da temperatura de transição vítrea que está em torno de 180ºC, a fim de prevenir a perda do material do recheio (DRUSCH et al., 2015). Além disso, de acordo com Drusch et al. (2015) diferenças na temperatura de secagem resultam em diferenças significantes na densidade das partículas contidas nas microcápsulas de MD.

Por outro lado, a goma arábica (GA) é o exsudato gomoso dessecado extraído do tronco de árvores do gênero *Acacia* da família Leguminosae (FDA, 2015). É definido como um polímero hidrocoloide multifuncional altamente ramificado por unidades de galactose, raminose, arabinose, ácido glicurônico e proteínas. O peso molecular da GA está em torno de 600.000 daltons e devido as ramificações, a GA possui forma globular. Esta estrutura faz como que a mesma seja útil na formação de revestimentos rígidos, favorecendo a proteção de óleos,

flavors, bioativos de origem vegetal e outros (CUNNINGHAM, 2011; ANANDHARAMAKRISHNAN, PADMA, 2015).

Em meio aquoso, a GA na concentração até cerca de 40%, se comporta como fluido Newtoniano, ou seja, a sua viscosidade não se altera com a taxa de cisalhamento. Em repouso ou em cisalhamento, a viscosidade permanece mais ou menos a mesma. No entanto, o aumento da temperatura faz com que a viscosidade do sistema diminua (CUNNINGHAM, 2011).

A GA contém três frações, uma conhecida como fração AGP (arabinogalactana-proteína), que inclui proteínas responsáveis pelas propriedades emulsificantes. A arabinogalactana contêm os grupos mais hidrofílicos que se orientam para a fase aquosa em uma emulsão enquanto os aminoácidos de cadeia lateral não polar da proteína são orientados para a hidrofóbica (CUNNINGHAM, 2011).

A superioridade da GA como agente encapsulante é demonstrada em vários estudos. Krishnan et al. (2005) compararam a eficiência da GA, MD e amido modificado como material de parede na encapsulação de oleoresina de cardamomo e observaram que a GA promoveu melhor proteção para a oleoresina comparado aos outros dois materiais, devido ao prolongamento da vida de prateleira do material encapsulado. Isto porque a plasticidade fornecida pela GA previne rachaduras da parede quando as partículas são submetidas à tensão durante a atomização e secagem (ANANDHARAMAKRISHNAN; PADMA, 2015). Por outro lado, Silva et al. (2013) verificaram que a MD apresentou maior eficiência na microencapsulação de extrato de jabuticaba em relação a retenção de antocianinas, higroscopicidade, manutenção da cor e atividade antioxidante. No entanto, MD (5%) juntamente com GA (25%) contribuiu para a formação de partículas mais homogêneas.

Ezhilarasi et al. (2014) atribuíram a MD o efeito protetor do ácido hidroxicítrico presente em extrato de *Garcinia cambogia*, que se manteve estável após ser exposto a altas temperaturas na secagem em spray dryer, além disso, observaram que a MD ofereceu uma alta eficiência na encapsulação do extrato. De acordo com Rosenberg *apud* Anandharamakrishnan e Padma (2015), a maltodextrina pode também atuar como um selante hidrofílico que limita a difusão do material encapsulado através do material de parede. Em adição a isso, a propriedade de formação da película, a MD também exibe alta digestibilidade, devido a sua estrutura bidimensional composta de simples unidades de glicose (ROSENBERG apud ANANDHARAMAKRISHNAN, PADMA, 2015)

Apesar dos benefícios citados acima, a MD apresenta falta de poder emulsificante reduzindo a estabilidade de compostos voláteis e óleos. No entanto, a combinação da MD com

outros materiais de parede com boa capacidade emulsificante tal como a GA, supera tal limitação (ANANDHARAMAKRISHNAN, PADMA, 2015).

6. Microencapsulação de extratos vegetais ricos em compostos fenólicos

Substâncias bioativas, oriundas do metabolismo secundário de vegetais, têm sido incorporadas a alimentos a fim de agregar valor aos alimentos industrializados e elevá-los a categoria de alimentos funcionais.

A extração de compostos naturais a partir de vegetais para a fabricação de produtos ricos em bioativos é de suma importância, tendo em vista que são altamente utilizados na produção de alimentos funcionais, produtos farmacêuticos, nutracêuticos e indústria cosmética. No entanto, devido à limitada estabilidade dos compostos fenólicos, uma das classes de bioativos mais estudadas quanto ao aspecto funcional, têm sido aplicadas várias técnicas de microencapsulação para proteger estes compostos (MUNIN; EDWARDS-LÉVY, 2011; AIZPURUA-OLAIZOLA et al., 2016), visando o aumento da vida de prateleira, o mascaramento de sabores desagradáveis, impedimento da interação destes compostos com macro e micronutrientes presentes nos alimentos, e a ampliação de seu uso em diferentes setores. Frente a isso, Aizpurua-Olaizola et al. (2016) microencapsularam resíduo de uva (*Vitis vinífera*), proveniente da fabricação de vinho, utilizando alginato de sódio polimerizado como material de parede. Os autores verificaram que os polifenóis microencapsulados apresentaram uma maior estabilidade comparado aos polifenóis livres.

Extrato rico em compostos fenólicos livre e microencapsulado de flores de amoras silvestre (*Rubus ulmifolius*) foram incorporados separadamente em formulação de iogurte com o propósito de atuar como bioativo com capacidade antioxidante. Os autores avaliaram os iogurtes em dois diferentes tempos (logo após a adição do extrato livre ou microcápsulas e 3 dias depois) e observaram que o extrato microencapsulado manteve uma maior atividade antioxidante quando comparado ao extrato livre ao ser adicionado nas formulações de iogurte (MARTINS et al., 2014). Outros estudos que avaliaram a interferência da microencapsulação sobre a vida de prateleira de extratos ricos em compostos fenólicos, concluíram que este método afeta positivamente na manutenção do extrato tanto em relação as propriedades físico-químicas quanto em relação as propriedades funcionais (MUNIN; EDWARDS-LÉVY, 2011; EZHILARASI, et al., 2014).

A fim de testar o efeito hipotrigliceridemiante e protetor do sistema cardiovascular do extrato de chá verde, Jung et al. (2013) administraram o extrato livre ou microencapsulado com

MD em ratos portadores de síndrome metabólica, induzida por uma dieta rica em frutose. Os autores verificaram que os animais tratados com as microcápsulas apresentaram menor dano no sistema cardiovascular, diminuição nos níveis séricos de triglicerídeos, colesterol e de ácido úrico e aumento da atividade da catalase hepática, devido a maior biodisponibilidade e a proteção dos compostos bioativos. Munin & Edwards-Lévy (2011) explicam que os polifenóis, devido à baixa solubilidade em sistemas aquosos, baixa estabilidade em condições alcalinas encontradas nos fluidos biológicos e baixa permeabilidade, frequentemente, apresentam baixa biodisponibilidade. Portanto, o processo de microencapsulação, além de manter a integridade estrutural do polifenóis até o consumo, mascara o sabor, aumenta a solubilidade em água e a biodisponibilidade e, ainda, melhora a eficácia e a segurança desses compostos.

Os ácidos fenólicos também podem interagir com aminoácidos, peptídeos e proteínas causando retardamento tanto da absorção dos ácidos fenólicos quanto dos aminoácidos, culminando em redução da capacidade antioxidante, especialmente quando o alimento é submetido ao aquecimento (RAWEL; ROHN, 2010). Além disso, a presença de ACGs em produtos alimentícios pode sofrer escurecimento enzimático e não enzimático nos alimentos e resultar em um sabor amargo (BUDRYN; RACHWAŁ-ROSIAK, 2013).

Diante dessas implicações Budryn et al. (2014) propuseram a extração, isolamento e a formação de um complexo de inclusão de ACGs do café verde com β-ciclodextrina. Nesse estudo os autores relataram um aumento da capacidade antioxidante com a formação do complexo. Em estudo posterior Budryn et al. (2015) verificaram a interação deste complexo com matrizes alimentares ricas em proteínas (isolados proteicos de ovo, soro do leite e soja) e a bioacessibilidade dos ACGs concluindo que os ACGs β-ciclodextrinizados são completamente liberados das matrizes alimentares (>99%) no intestino e, portanto, altamente bioacessíveis.

7. Café verde

Atualmente, o café verde tem sido matéria prima para a produção de extratos secos que são utilizados para fins de emagrecimento (FLANAGAN et al., 2014), como adjuvante no tratamento de síndromes metabólicas como o diabetes mellitus tipo II (ONG; HSU; TAN, 2013) e dislipidemias (RODRIGUEZ DE SOTILLO; HADLEY, 2002) e na prevenção de doenças degenerativas como o câncer, doenças cardiovasculares e mal de Parkinson (HIGDON; FREI, 2006). Consequentemente, houve um aumento no interesse em caracterizar compostos

bioativos do café que podem funcionar como agentes fisiologicamente ativos. Os ACG, juntamente com a cafeína, são considerados as principais classes de compostos bioativos (LUDWIG et al., 2014).

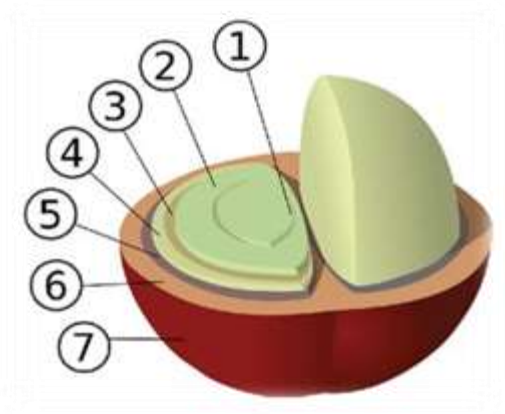
Estudos científicos que avaliaram a concentração de ACG em frutos imaturos do café verificaram que o fruto, contendo a casca e a polpa, mantêm maior proporção destes compostos bioativos quando comparados aos grãos verdes despolpados (KOSHIRO et al., 2007; NOBRE et al., 2011). Além disso, quanto mais verde estão os frutos (estágios I e II), maior é proporção de ACG de acordo com KOSHIRO et al. (2007).

Por outro lado, Rossetti (2007) observou que a quantidade de polifenóis totais foi maior na semente do que na casca em função da proteção da semente, tendo em vista que os compostos fenólicos presentes no café fazem parte do sistema de proteção via metabolismo secundário como fitoanticipinas (que são de defesa constitutiva). Além disso, verificou-se que na etapa final de maturação do fruto, as sementes apresentam uma redução na quantidade de polifenóis.

8. Composição nutricional e fitoquímica do café verde

O fruto do café (Figura 3) é constituído por casca, polpa, mucilagem, pergaminho e semente (ROSSETTI, 2007). O mesmo apresenta alta concentração de carboidratos (~60% em base seca) incluindo polissacarídeos solúveis e insolúveis (celulose, arabinogalactana e galactomanana), oligossacarídeos (estequiose e rafinose), dissacarídeo (sacarose) e monossacarídeos (glucose, galactose, frutose, manose, manitol, xilose e ribose), sendo que a quantidade de polissacarídeos no café Robusta é ligeiramente maior do que a do café Arábica (LUDWIG et al., 2014). O teor de lipídeos do café verde é em torno de 8 a 18% em peso seco, sendo que o café arábica apresenta uma concentração de lipídeos significantemente mais elevada que o café robusta (KURZROCK; SPEER, 2001).

De acordo com Kurzrock e Speer (2001), a fração lipídica do café consiste de 75% de triglicerídeos e os demais 25% é constituído de esterol (estigmasterol, sistosterol), ácidos graxos (oleico, linoleico, linolênico, palmítico, esteárico, eicosanóico, tetracosanóico e docosanóico) e diterpenos pentacíclicos (cafestol, caveol). Proteínas, peptídeos e aminoácidos representam em torno de 9 a 16% em base seca do café verde. Os principais aminoácidos, tanto ligado a proteína quanto livres, são a asparagina, ácido glutâmico, alanina, ácido aspártico e lisina (LUDWIG et al., 2014).



1 – Corte central

2 – Grão (endosperma)

3 – Película prateada (espermodema)

4 – Pergaminho (endocarpo)

5 – Mucilagem

6 – Polpa (mesocarpo)

7 – Película exterior (exocarpo/pericarpo)

Figura 3. Anatomia do fruto do café (Wintgens, 2012).

Além dos compostos fenólicos, outros metabólitos de importância tecnológica e biológica presentes no café são a cafeína, alcaloide pertencente ao grupo das xantinas com atividades farmacológicas reconhecidas cientificamente e outras metilxantinas, tal como, a teobromina, teofilina e trigonelina. Presente em alta concentração nos frutos do café, a trigonelina, uma N-metil betaína, é muito importante para o sabor e aroma do café, pois durante a torra do grão, esta molécula contribui com a formação de produtos de degradação que fornecem o aroma característico do café, dentre eles estão a piridina e o N-metilpirrol (MONTEIRO; TRUGO, 2005).

O café verde também contém uma variedade de polifenóis com quantidade variando entre 6 a 10% do peso seco, sendo esta quantidade maior no café robusta que no café arábica (LUDWIG et al., 2014). Os ácidos fenólicos são os mais abundantes no grão imaturo (SOGA; OTA; SHIMOTOYODOME, 2013) e os ácidos clorogênicos, especialmente o ácido 5-cafeoilquínico, éster de ácido quínico e de ácidos *trans*-hidroxicinâmicos, são os majoritários. Em menor quantidade estão os ácidos feruloilquinico e dicafeoilquinico, sendo que estes são relacionados estruturalmente por mais de 50 outros ácidos cinamoilquínico e alguns conjugados de amino-cinamoil e cinamoil-glicosídeos (Figura 4) (MEHARI et al., 2016). Os ácidos hidroxicinâmicos incluem os ácidos *p*-coumarico, cafeico, sinapínico, ferúlico e são geralmente encontrados em plantas comestíveis (BALASUNDRAM; SUNDRAM; SAMMAN, 2006).

O perfil de ácidos fenólicos no café depende principalmente da variabilidade genética, práticas agronômicas e fatores ambientais e, em menor grau de importância, condições de colheita e pós-colheita (MEHARI et al., 2016; RODRIGUES et al., 2015).

9. Atividades biológicas relacionadas ao café e seus constituintes

Considerando a enorme demanda pelos produtos do café, o efeito do seu consumo sobre a saúde tem demandado atenção em relação às questões de saúde pública. Um elevado número de estudos correlacionando café e a sua interferência com a saúde foram realizados nas últimas décadas. De acordo com Wierzejska (2015), até recentemente, o consumo do café estava sendo associado com inúmeros efeitos adversos, principalmente ansiedade e problemas cardiovasculares. Por outro lado, resultados de pesquisas apresentados no artigo de revisão publicado por Hermansen et al (2012) enfatizam que o consumo do café pode, de fato, ser um elemento benéfico a ser incorporado na dieta.

À luz do conhecimento atual, sabe-se que o consumo moderado do café pode ajudar a prevenir doenças crônicas como o diabetes mellitus tipo II, doenças cardiovasculares e doença de *Parkinson.* Estes benefícios estão ligados aos metabolitos secundários do café, da classe das xantinas, tal como a cafeína e a trigonelina (LIAO et al., 2015) e, principalmente, aos efeitos biológicos atribuídos aos ACGs (MENG et al., 2013; REVUELTA-INIESTA, DAJUAILI, 2014).

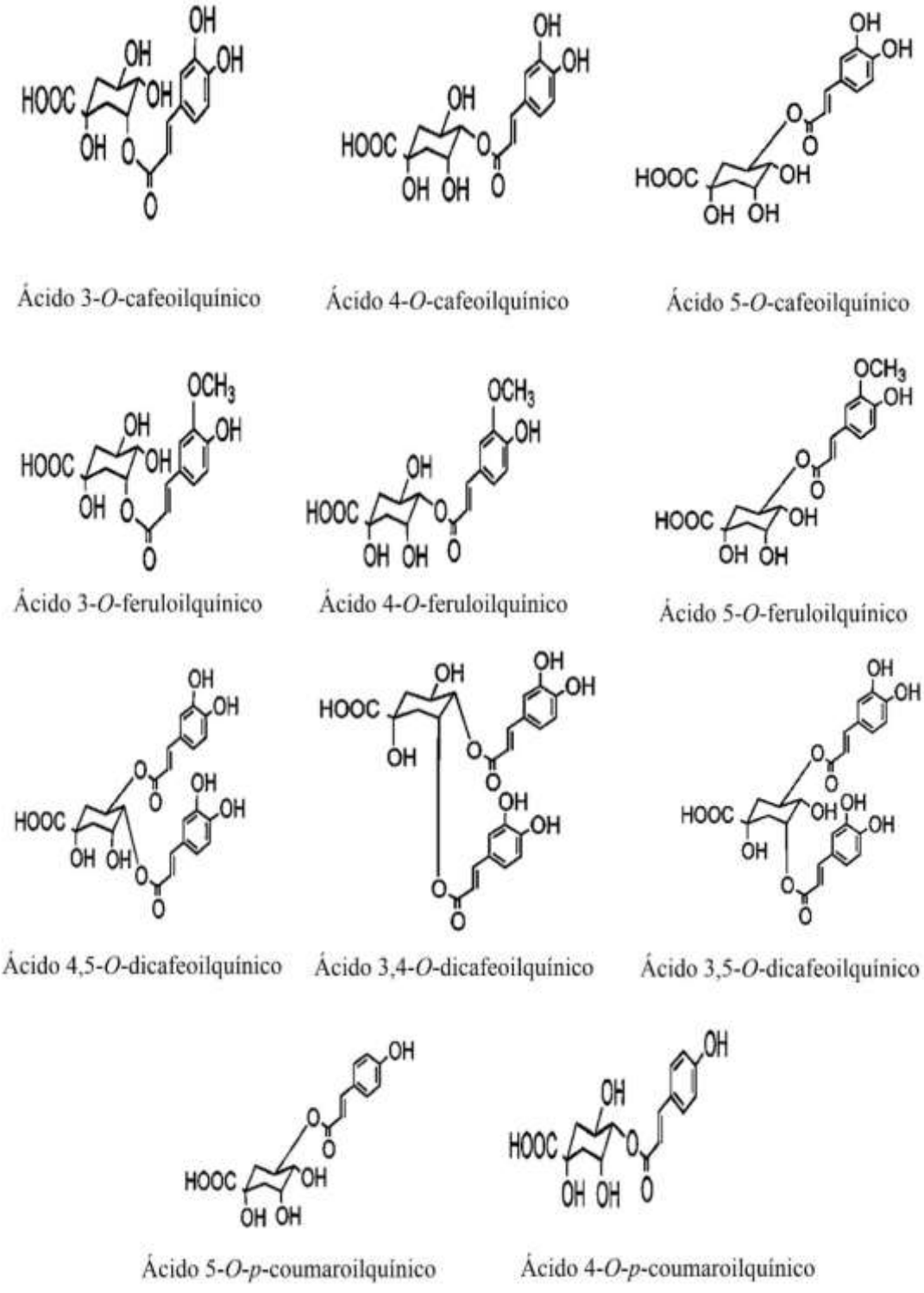


Figura 4. Estrutura dos principais ACGs encontrados no café verde (LUDWING et al., 2014).

9.1 Capacidade antioxidante e neuroprotetora

O desenvolvimento de doenças crônico-degenerativas está intimamente ligado com o acúmulo excessivo de espécies reativas de oxigênio e de nitrogênio nos fluidos biológicos incluindo radicais livres, tal como o ânion superóxido, radical hidroxila, radical hidroperoxila.

Outra problemática gerada com o desbalanceamento oxidativo é a oxidação das lipoproteínas de baixa densidade, que podem se depositar nas paredes dos vasos sanguíneos levando a aterosclerose e o desenvolvimento de doenças cardiovasculares. Além disso, o estresse oxidativo desempenha um importante papel na patogênese do envelhecimento (ASMAT; ABAD; ISMAIL, 2015).

De acordo com Nemzer et al. (2013), o estresse oxidativo pode ser reduzido através de terapia antioxidante que nos últimos anos tem ganhado o interesse na prática médica. Além de medicamentos e suplementos dietéticos com efeitos antioxidantes, os alimentos e bebidas, vem ganhando interesse crescente, principalmente àqueles considerados “superalimentos”. Frente a isso, vários bancos de dados foram criados, trazendo informações sobre o conteúdo de polifenóis e antioxidantes nos alimentos (CARLSEN et al., 2010; YASHIN et al., 2010). O estudo realizado por Carlsen et al. (2010) consistiu na medida da atividade antioxidante em mais de 3100 alimentos, bebidas, especiarias, ervas e suplementos dietéticos utilizados mundialmente por meio do método FRAP (poder antioxidante por redução do íon férrico). Os resultados deste trabalho demonstraram que a bebida de café apresenta conteúdo de substâncias com capacidade antioxidante significativamente maior quando comparado ao vinho tinto, chá, suco de romã e outras bebidas analisadas.

Em diferentes estudos o extrato obtido do grão verde ou torrado do café, tem apresentado eficácia tanto em relação a capacidade antioxidante *in vitro* e *ex vivo* (DAGLIA et al., 2004; RODRIGUES, BENASSI, BRAGAGNOLO, 2014; RODRIGUES, SALVA, BRAGAGNOLO, 2015) quanto relacionado a capacidade antioxidante *in vitro* (CARLSEN et al., 2010; BOETTLER et al., 2011). Devido a isso, o consumo do café e seus componentes têm sido associado ao menor risco do desenvolvimento de câncer, trombose e outras doenças crônico-degenerativas, além de protetor tecidual. De acordo com Boettler et al. (2011), esta proteção pode ser explicada pela ação do café interferindo na ativação do fator nuclear eritroide 2 relacionado com o fator 2 (Nrf2) uma vez que este fator de transcrição desempenha um papel crítico na regulação da expressão de genes que codificam a síntese de agentes detoxificante da fase II e enzimas antioxidantes, tal como a superóxido desmutase, catalase e heme-oxigenase 1 (BOETTLER et al., 2011).

Li et al. (2008) também encontraram relação entre o 5-CQA e a proteção neuronal contra apoptose por estresse oxidativo induzido pela exposição de células PC12, oriundas da linhagem clonal de feocromocitoma que exibem características de neurônio, ao metilmercúrio (MeHg). Os autores atribuíram o efeito neuroprotetor ao 5-CQA devido ao seu papel na capacidade desativadora de espécies reativas de oxigênio, decréscimo na atividade de glutationa peroxidase e no conteúdo de glutationa reduzida bem como pela atenuação da ativação da caspase-3 que desempenha um papel central na fase de execução da apoptose celular.

Segundo Pietro e Vásquez (2014), estudos recentes têm sido desenvolvidos com o objetivo de avaliar as alterações no conteúdo de ACGs em diferentes espécies de cafés, cultivadas em diferentes localizações e climas visto que setores industriais como o farmacêutico e alimentício têm apresentado interesse pela extração destes compostos, mais proeminentes no café cru, devido a sua capacidade antioxidante já comprovada em diversos estudos (DAGLIA et al., 2004; CARLSEN et al., 2010; BOETTLER et al., 2011).

Pietro e Vásquez (2014) analisaram a capacidade antioxidante de extratos hidrofílicos e lipofílicos de cafés crus (Robusta e Arábica), provenientes de 5 países com diferentes climas utilizando o teste da descoloração dos carotenoides β-caroteno e crocina, os mesmos concluíram que os extratos hidrofílicos de ambas as espécies apresentam maior capacidade antioxidante quando comparado aos extratos lipofílicos das mesmas espécies.

Em estudo realizado por Rodrigues et al. (2015) foi avaliada de forma inédita a influência do genótipo do café sobre a capacidade desativadora de espécies reativas de oxigênio e de nitrogênio *in vitro.* Os resultados deste estudo mostraram que os extratos hidrofílicos de café verde da espécie *C. canephora* e de *C. kapakata* foram os mais eficientes na desativação do $ROO^{\bullet}$, H_2O_2, HOCl, $NO^{\bullet}$ e $ONOO^{-}$, tendo em vista que apresentaram teores de ácidos clorogênicos em média 30% superiores aos encontrados em outras espécies como *C. arabica* e *C. racemosa.*

Alguns estudos têm buscado avaliar a possibilidade da utilização do extrato de café cru como suplemento para produção de alimentos funcionais ou nutracêutico. Neste sentido, Glei et al. (2006) avaliaram a capacidade antioxidante, pelo método FRAP, de pães enriquecidos com extrato de café verde ou cru em células do colón (HT29) e do fígado (HepG2), bem como, a citotoxicidade e genotoxicidade do pão enriquecido sobre as células através do teste de cometa. Em suma, o pão enriquecido com extrato do gão cru do café não apresentou efeito genotóxico ou citotóxico nas linhagens celulares e, em contrapartida, aumentou as propriedades

quimioprotetivas bem como a resistência das células do cólon e fígado contra os danos ao DNA induzidos por H_2O_2 no teste de cometa (GLEI et al., 2006).

Estudo preliminar realizado por Dziki et al. (2015) avaliou a possibilidade da utilização do extrato do grão cru do café como um suplemento alimentício funcional por meio de ensaios *in vitro* que avaliaram a digestão e absorção dos compostos antioxidantes do grão cru do café, capacidade antioxidante da fração bioacessível pelos ensaios de inibição da peroxidação do ácido linoleico catalisada pela hemoglobina, capacidade de captura de radicais pelos ensaios de ABTS e FRAP e análise sensorial de pão enriquecido com o grão de café cru. Os mesmos obtiveram êxito em ambos os ensaios e ressaltaram a importância do consumo do grão do café cru na sua totalidade, pois outros constituintes do fruto, tal como a cafeína, apresentam efeitos positivos na proteção celular, além de contribuir com um maior gasto energético devido ao seu efeito termogênico e na melhora de humor (DZIKI et al., 2015).

O uso de extratos obtidos das sementes do café cru como agente antioxidante tem sido avaliado em alguns estudos. O efeito antioxidante do extrato de soja enriquecido com extrato obtido do café cru foi estudado por Sęczyk et al. (2017) que verificaram um aumento dose dependente da atividade antioxidante da mistura. Budryn et al. (2014b) observaram que a adição do extrato obtido da semente do café cru em óleo de girassol puro ou adicionado de outros ingredientes usados em produtos de confeitaria tal como amido, sacarose e proteína da clara do ovo diminuiu a perda dos ácidos graxos poli-insaturados presentes no óleo de girassol e aumentou a estabilidade antioxidante de ambos os sistemas, mesmo após aquecimento. Świeca et al. (2017) obteve bons resultados ao suplementar pães de trigo com extrato das sementes do café cru, além de aumentar o potencial nutracêutico. De acordo com os autores, os compostos fenólicos do café cru, bem como a cafeína, preveniram a peroxidação lipídica no pão.

Estudos epidemiológicos têm mostrado a correlação entre o consumo do café e os efeitos neuroprotetivos e de prevenção de doenças neurodegenerativas como *Parkinson* pela capacidade antioxidante desempenhada pelos seus constituintes. Em estudo realizado por Daglia et al. (2004), culturas de células de neuroblastoma humano (IMR 32) foram expostas a soluções contendo café verde e todas apresentaram capacidade antioxidante promovendo maior sobrevida das células em meio oxidante, sendo que as frações mais ativas apresentaram uma dose-resposta linear em relação a concentração de ácido 5-cafeioilquínico (5-CQA).

Em estudo piloto conduzido por Reyes et al. (2013a), dose única de extrato seco obtido do fruto inteiro do café cereja ou extrato do grão cru do café rico em cafeína ou extrato de sementes da uva (100 mg/dose), resultou em um aumento 4,6 maior no nível do fator neurotrófico derivado do cérebro (BNDF, do inglês *Brain-derived neurotrophic fator*), proteína

que ajuda na sobrevivência neuronal e na neurogênese, em indivíduos que consumiram o extrrato do café cereja em comparação aos voluntários que receberam o extrato do grão do café cru ou extrato da semente de uva. Posteriormente, Reyes et al. (2013b) realizaram um estudo controlado por placebo, no qual 20 voluntários saudáveis receberam placebo no dia 1, dose única de extrato do fruto inteiro da café cereja (100 mg/dose) no segundo dia e 300 mL de bebida do café no terceiro dia. O tratamento com extrato de café cereja resultou em aumento da BNDF significativo em relação ao placebo e bebida do café nos primeiros 60 minutos após a administração do mesmo.

Frente a esses resultados, o desenvolvimento de extratos a partir do grão e/ou fruto do café cru poderá levantar um mercado ainda mais promissor ao mercado cafeeiro frente a indústria alimentícia e farmacêutica.

9.2 Atividade anticancerígena

Estudos sobre a atividade anticancerígena ligada ao consumo da bebida do café foram recentemente revisados por Wierzejska et al. (2015) que demonstraram que existem resultados controversos. De acordo com os autores, apesar do café conter numerosos compostos de efeito protetor, tal como, os polifenóis que inibem o processo de oxidação no organismo, as substâncias formadas durante a torrefação do grão, principalmente pela reação de *Maillard* entre o aminoácido asparagina e os açucares redutores, culmina na produção de substâncias cancerígenas tal como a acrilamida.

Neste sentido, na década de 80, Snowdon e Phillips (1984) associaram o consumo do café com o aumento do risco de desenvolvimento de câncer colorretal. Por outro lado, uma meta-análise de estudos de coorte publicada em 2011, concluiu que o consumo do café diminui a incidência do desenvolvimento do câncer colorretal (YU et al., 2011). Assim como em um estudo de caso-controle realizado por Li et al. (2013) no qual os autores averiguaram que o risco de desenvolvimento de câncer colorretal e de cólon foi reduzido em 15% e 21%, respectivamente, em consumidores assíduos de café quando comparado a não consumidores ou consumidores menos assíduos.

Ruan et al. (2014) verificaram o papel de proteção intestinal dos ACG em ratos Sprague-Dawley. Neste ensaio, os pesquisadores induziram a inflamação intestinal nos animais administrando lipopolissacarídeo bacteriano e observaram que os grupos suplementados com 20 e 50 mg/kg de ACG apresentaram uma melhora na estrutura intestinal, diminuindo danos e aumentando a integridade da mucosa intestinal pela atenuação da disfunção na zônula de

oclusão do epitélio intestinal devido ao aumento da síntese de proteínas ZO-1 e ocludinas intestinal. Este pode ser um dos mecanismos responsável pela proteção colorretal e consequentemente diminuição do risco do desenvolvimento de tumores malignos nesta região (RUAN et al., 2014).

9.3 Atividade antidiabética e antiobesogênica

O café, por sua riqueza em compostos bioativos, tem sido relacionado com inúmeros benefícios à saúde. Estudos sobre os efeitos tanto da bebida do café torrado (REVUELTA-INIESTA; AL-DUJAILI, 2014) quanto do grão do café cru (LI KWOK CHEONG et al., 2014) na saúde humana foram realizados. Estes efeitos são frequentemente relacionados à cafeína, um alcaloide farmacologicamente ativo, pertencente ao grupo das xantinas, com efeitos farmacológicos já comprovados cientificamente tal como estimulantes do sistema nervoso central, estimulante do músculo cardíaco (MONTEIRO; TRUGO, 2005); melhora na performance durante o exercício, particularmente devido a sua atividade no sistema neuromuscular e na ressíntese do glicogênio após o exercício (BEAM et al., 2015). Devido ao seu efeito estimulante e termogênico, tem sido atribuído à cafeína efeitos na redução do peso e, consequentemente, redução do risco de desenvolvimento de síndrome metabólica (DZIKI et al., 2015).

A cafeína ou 1,3,7-trimetilxantina e outras metilxantinas presentes no café como a teofilina e teobromina atuam causando a lipólise dos adipócitos, através da inibição da fosfodiesterase e aumentando a adenosina monofosfato cíclica (AMPc) por meio da inibição dos receptores de adenosina. O aumento nos níveis de AMPc, ativa as lipases hormônios sensíveis, promovendo a lipólise (PANCHAL et al., 2012).

Embora a cafeína contribua com alguns efeitos benéficos atribuídos ao café, uma meta-análise envolvendo mais de 500.000 indivíduos correlacionou um menor risco de desenvolvimento do DM tipo II com o consumo de café descafeinado, sugerindo que outros componentes bioativos presentes no café possuem mecanismos de ação protetores contra o risco de desenvolvimento de doenças de cunho metabólico tal como a obesidade, hipertensão e DM tipo II (HUXLEY et al., 2006; MENG et al., 2013; REVUELTA-INIESTA; AL-DUJAILI, 2014). Estes efeitos são altamente relacionados com os ACG, os quais demonstraram estimular os receptores alfa ativados por proliferador de peroxissomo (PPAR-α) e aumentar a β-oxidação de ácidos graxos (CHO et al., 2010; SOGA; OTA; SHIMOTOYODOME, 2013). Além disso, estão relacionados na diminuição do nível de triacilgliceróis e do colesterol plasmático durante

o jejum, bem como a concentração de triacilgliceróis no tecido hepático (RODRIGUEZ DE SOTILLO; HADLEY, 2002) e no aumento da a sensibilidade periférica à insulina culminando com uma maior utilização celular de glicose (PHAM et al., 2014).

Yinan et al. (2007) indicaram que o ACG extraído da folha do café atua inibindo as enzimas α-glicosidase e α-amilase nas células intestinais favorecendo a diminuição da glicemia pós-prandial em ratos. Este efeito foi comparável ao da acarbose, medicamento que age inibindo fortemente a α-glicosidase intestinal.

De acordo com Meng et al. (2013), o mecanismo pelo qual os ACGs exercem efeito antidiabético se dá via estímulo do consumo de glicose tanto em adipócitos insulinorresistentes quanto adipócitos sensíveis a ação da insulina. Ensaios *ex vivo*, com culturas celulares de adipócitos, demonstraram que os ACGs foram mais potentes em estimular o consumo de 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-d-glucose (2-NBDG), um análogo fluorescente da glicose, comparado a droga antidiabética rosiglitazona (ALONSO-CASTRO et al., 2008).

Tsuda et al. (2012) exploraram os efeitos hipoglicemiantes do ACG e seu metabólito, o ácido cafeico, no tecido muscular esquelético de ratos e observaram que o ácido cafeico, mas não o ACG, atua diretamente estimulando a síntese da proteína quinase ativada por adenosina monofosfato (AMPK) essa, por sua vez, estimula a síntese de transportadores de glicose GLUT4 independente de insulina no músculo esquelético estimulando o consumo de glicose por estas células.

Em um estudo transversal, Pham et al. (2014) investigaram a associação do consumo habitual do café com a resistência à insulina. Participaram deste estudo 1665 indivíduos, os quais se submeteram a exames de glicemia e insulinemia de jejum, avaliação homeostática de células β (HOMA-β) e avaliação homeostática de resistência à insulina (HOMA-IR). Os resultados deste estudo sugerem uma correlação positiva do consumo do café com a diminuição da resistência à insulina, mas não com o aumento da secreção da insulina pelas células da ilhota pancreática.

O ACG se mostrou eficaz também na prevenção de complicações microvascular do DM, tal como a retinopatia diabética. Shin et al. (2013) analisaram o efeito protetor vascular do ACG em modelo animal de retinopatia diabética. Os ratos Sprague-Dawley foram tratados com 10 ou 20mg/kg dia de ACG intraperitonealmente por 14 dias e verificaram que o ACG, de forma dose-dependente, pode ter um papel na prevenção da ruptura da barreira hemato-retiniana por preservar as proteínas da zônula de oclusão do epitélio vascular e por diminuir o nível do fator

de crescimento endotelial vascular que está diretamente ligada com o aumento na permeabilidade vascular e com a neovascularização patológica (SHIN et al., 2013).

Um dos principais fatores ambientais relacionados com o desenvolvimento do DM tipo II é a obesidade. Assim, Onakpoya et al. (2011) fizeram um levantamento sobre ensaios clínicos randomizados que avaliaram a eficácia do ECV como suplemento dietético para a perda de peso. Neste trabalho foram incluídos três estudos nos quais indivíduos com sobrepeso receberam dosagens de 180 mg de ECV diariamente por 4 semanas (AYTON GLOBAL RESEARCH, 2009) e 200 mg de ECV diariamente por 12 semanas (DELLALIBERA; LEMAIRE; LAFAY, 2006; THOM, 2007). Com base nos resultados obtidos os autores indicaram que o consumo do ECV pode promover perda de peso, no entanto, o efeito demonstrado em ambos os estudos não foi conclusivo e a relevância clinica deste efeito foi incerto, o que remete à necessidade de estudos mais extensos para obtenção de resultados confiáveis.

Produto nutracêutico livre de prescrição médica a base de ECV com elevada concentração de ACGs também foi avaliado em estudo transversal, randomizado, duplo-cego, placebo-controlado em indivíduos com sobrepeso, não obesos. Dezesseis indivíduos receberam 1050 mg/kg, 700 mg/kg de ACG e placebo, no seguinte esquema: sequência de alta dose/baixa dose e placebo (n=6), sequência de baixa dose, placebo e alta dose (n=4) e sequência de placebo, alta dose e baixa dose (n=6) por 22 semanas com intervalo de 2 semanas para cada tratamento. Os indivíduos foram avaliados em relação ao peso, percentagem de gordura corporal, índice de massa corpórea, pressão arterial sistólica e diastólica e frequência cardíaca. Os resultados deste estudo mostraram que 10 dos 16 participantes apresentaram cerca de 10% de perda de peso, sendo que outros 5 restantes tiveram uma perda de peso em média de 5%. Em relação à pressão arterial, os participantes tiveram um aumento não significativo na pressão arterial sistólica (VINSON; BURNHAM; NAGENDRAN, 2012).

Em estudo mais recente Flanagan et al. (2014) avaliou o efeito lipolítico, em adipócitos isolados do tecido abdominal humano, do Svetol®, um ECV descafeinado, comercializado pela empresa Naturex Inc., utilizando a cafeína como controle-positivo. A atividade lipolítica foi expressa em µM de ácidos graxos livre liberados no meio. Com base nos resultados, os autores observaram que a cafeína apresentou um forte efeito lipolítico, 67% maior comparado ao Svetol®. Neste sentido, os autores atribuíram grande parte do efeito lipolítico do ECV à cafeína presente, provavelmente, de forma remanescente, nos extratos utilizados em estudos prévios.

10. Referências

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CAPITULO III

An Optimized Green and Economical Extractive Method to Obtain Bioactive Compounds from Green Coffee Fruits

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ABSTRACT

Green coffee fruit is a main source of chlorogenic acids, caffeine and trigonelline, compounds which have been widely associated with health benefits, suggesting the potential application of green coffee fruit in the pharmaceutical, food and cosmetic industries. Thus, the present research aimed to optimize the extraction of bioactive compounds from green coffee fruits using a green and economical method such as percolation, by way of a 2^2 central composite rotatable design (CCRD) and response surface methodology (RSM). It also aimed to validate an HPLC-DAD-MS analytical method for the determination of chemical markers in the dried green coffee fruit extract (GCFE) produced on a large scale and monitor the stability of the extract for 180 days. The two independent variables studied in the 2^2 CCRD were the ethanol concentration (% w/w) and solid to solvent proportion (w/w). The 5-caffeoylquinic acid (5-CQA), caffeine and trigonelline contents ($mg.1000g^{-1}$) and the process yield were the dependent variables assessed. The HPLC-DAD-MS analytical method used to determine the 5-CQA, caffeine and trigonelline contents proved to be selective, linear, precise, accurate and robust. Based on the response surface, the best conditions for the extraction of 5-CQA by percolation were an ethanol concentration in the range from 65 – 80 % (w/w) and a solid to solvent proportion in the range from 0.8:10 – 1:10 (w/w). The dried GCFE was found to be stable for 180 days at 40°C, which suggests its potential application in the pharmaceutical, dietary (e.g. sports nutrition supplement), functional food, food additive and cosmetic industries.

Keywords: 5-caffeoylquinic acid (5-CQA), HPLC-DAD-MS, spray dried extract, reducing capacity, methylxanthines, accelerated stability.

1. Introduction

In recent years, interest in the development of methods to extract biologically active metabolites from plants has grown considerably, particularly in the medicinal chemistry area [1]. Chlorogenic acids (CGA) are phenolic compounds found in all higher plants. Structurally, CGA are esters formed from quinic acid and certain *trans*-cinnamic acids, mainly caffeic, ferulic and *p*-coumaric acid [2], green coffee fruit being the major source of CGA [3,4]. Since hydroxyhydroquinone compounds, that can inhibit the actions of CGA *in vivo*, are formed during the roasting of coffee beans, the use of green coffee is preferable to the use of the coffee beverage [5,6]. According to Nobre et al. [7], the whole coffee fruit presents a large amount of CGA and not just the beans, and besides, Koshiro et al. [8] found that the CGA content was greater in first stages I and II of fruit maturation than in stages III and IV, and the amounts were significantly lower in stage V.

Of the chlorogenic acid family, 5-caffeoylquinic acid (5-CQA) is the most studied compound [9], and innumerous studies concerning the bioactivity of 5-CQA have suggesting its potential application in the pharmaceutical, dietary (functional food, food additive) and cosmetic industries [3,4, 10–13]. Other phytochemical compounds found in green coffee fruit, such as the methylxanthines trigonelline [14], theophylline [15] and caffeine [16], and hydroxycinnamic acids such as caffeic [17,18] and ferulic [19] acids have also been associated with health benefits. Thus the whole green coffee fruit is a promising source of bioactive compounds to be applied in the development of a standardized vegetable extract to be used as a supplement in functional foods and sport nutrition supplements.

Optimization of the extraction method is highly correlated with the quality of the extract and the precision of the analytical methods applied to the bioproducts [20]. Many previous studies have focused on improvement of the extraction of 5-CQA from different vegetable matrices using different methods, such as the application of ionic liquid based enzyme-assisted extraction [21], air-steam explosion [22], ultrasound-assisted extraction [23], microwave-assisted extraction [24] and others [1,25]. However, classical methods as percolation are highly important to obtain a greater product yield at low cost for application in industrial processes [26]. Percolation consists of three distinct processes: wetting, extraction and hydrolysis. The efficiency of percolation extraction and its reproducibility depends of the particle size, solvent to solid proportion, flow rate, and the percentage of ethanol in the solvent [26]. However, there are no studies that show the impact of the percolation method on the quality and standardization of the green coffee extract from the whole fruit.

The lack of complete standardization for the formulation of herbal products is a great challenge due to variations in the manufacturing processes [27]. Pure phytoconstituents and plant extracts are rich in chemical diversity, implying in a complicated quality control process, since the compositions may be influenced by multiple factors, such as the origin, growth, harvesting, drying and storage conditions [28,29].

Thus in order to determine the optimal conditions to produce a green coffee fruit extract (GCFE) with standard amounts of caffeine, trigonelline and chlorogenic acids expressed as 5-CQA, and with a reproducible yield, an experimental design was applied to determine the influence of the solvent to solid proportion and the percent of ethanol on the hydroethanolic solvent. In addition the physical and chromatographic profiles of the standardized extract were characterized to ensure a high quality standardized extract.

2. Experimental

2.1 Chemicals and reagents

Chromatograph grade methanol was supplied by J.T. Backer (Phillipsburg, NJ, USA), analytical grade 99.5% ethanol was supplied by Synth (Diadema, SP, Brazil), citric acid was supplied by Quemis (Joinvile, SC, Brazil). Ultrapure water was obtained from a Millipore system (Billerica, MA, USA), and 5-caffeoylquinic acid (95% pure), caffeine (99% pure) and trigonelline (98.5% pure), supplied by Sigma-Aldrich (St. Louis, MO, USA), were used as the external standards.

2.2 Apparatus and equipment

The percolation extractions (PE) were carried out using a stainless steel percolator with a 500 mL capacity (P & L Ltda, Curitiba-PR, Brazil) and the extracts were concentrated using a Buchi® rotary evaporator (model R-220 SE). The quantitative analysis was carried out using a high performance liquid chromatograph (HPLC) (Shimadzu HPLC, Kyoto, Japan) equipped with a binary pump (LC-10AD), degasser with helium (DGU-2A), automatic injection system (SIL-10A) and a diode array detector (SPD-M-10A). Chemical markers for the 5-CQA, chlorogenic acid derivatives and methyxanthines were identified in a HPLC (Shimadzu HPLC, Kyoto, Japan) equipped with a quaternary pump (LC-20AD), online degasser (DGU-20A5), rheodyne injection valve with a 20 mL loop, diode array detector (Shimadzu, SPD-M20A) and a mass spectrometer detector with an ion trap analyser and electrospray ionization (ESI) source (Bruker Daltonics, model Esquire 4000, Bremen, Germany). The chromatographic separations

were carried out using a Shim-pack ODS-C_{18} column (5 µm, 250 x 4.6 mm, Shimadzu, Kyoto, Japan) coupled to an ODS-C18 pre-column (5 µm, 4 x 3 mm, Phenomenex, Torrance, CA, USA).

2.3 Plant material

Fresh *C. canephora* (clone code 83), green fruits were acquired from the Experimental Farm of the company EPAMIG in Leopoldina (Minas Gerais, Brazil), located at 21°28'.51.38" south latitude, 42°43'17.27" west longitude and 187 meters of altitude. Immediately after harvest the fruits were washed, selected, vacuum packed (Jumbo Plus, Selovac, SP, Brazil), and stored frozen protected from the light at -80ºC until extraction.

Before extraction the whole green fruits were dried at 40ºC in a circulating air oven until reaching 20% of moisture content. They were then crushed in a high performance blender model OBL 10/2 (Oxy, PB, Brazil) to pass through a 0.5 mm sieve, and vacuum packed in polyethylene bags. The pharmacognostic characterization of the sample was carried out according to the Brazilian Pharmacopeia [30], and showed a moisture content of 17.55 ± 0.39 g/100g, total ash content of 5.30 ± 0.09 g/100g, intumescence index of 2.1 ± 0.1 mL, water activity of 0.7 ± 0.0 and moderately coarse particle size of < 710 µm.

2.4 Determination of the extract compositions by HPLC-DAD-MS^n

The different extracts obtained using the optimized process were analysed according to Rodrigues et al. [31] whereby 5 mg of each extract was suspended in 1250 µL of a methanol: water (80:20 v/v) solution, the mixture vortexed for 30 s, and centrifuged at 25000*g* for 10 min at 10ºC. The supernatant was filtered through a 0.45µm membrane (Millipore, São Paulo, Brazil) and injected into the HPLC-DAD or HPLC-DAD-MS^n.

The quantitative analysis of the 5-CQA, caffeine and trigonelline was carried out using a mobile phase of 80% (v/v) 10 mM citric acid (pH 2.5) and 20% (v/v) of methanol (solvent A) and methanol (solvent B), with gradient elution [31]. The flow rate was 1 mL min^{-1} and the column oven temperature was maintained at 30 °C. The detection wavelengths were set at 262, 272 and 325 nm and external analytical curves with six points ranging from 99.8 to 1742.05 µg.mL^{-1} (R^2 = 0.99, $p < 0.05$), 37.21 to 452.16 µg.mL^{-1} (R^2 = 0.99, $p < 0.05$) and 39.95 to 746.43 µg.mL^{-1} (R^2 = 0.99, $p < 0.05$), respectively, in triplicate, were used to quantify the 5-CQA, trigonelline and caffeine, respectively.

The caffeine, trigonelline and chlorogenic acids and their derivatives, present in the extract were identified using the same chromatographic conditions as described for the quantification analyses, but substituting the citric acid in solvent A by 0.3% formic acid (pH 2.5), since citric acid generates fragments at *m/z* 191, which interfere in compound identification [34]. The drying gas flow (N_2) was 8 L/min, the nebulizer pressure 30 psi, and the drying gas temperature 310 °C. For electro spray ionization (ESI), the following conditions were applied: positive ionization mode for the identification of caffeine, trigonelline and theophylline, and the negative ionization mode for the identification of 5-CQA and other chlorogenic acid derivatives, with a capillary voltage of 2500 V and fragmentation energy of the MS^2 and MS^3 = 1.6 V. Mass spectra were acquired in the range from 70 to 600 *m/z*. Trigonelline, caffeine and 5-CQA were identified according to the elution order on the reversed phase column, and the UV-visible and mass spectra characteristics as compared to those obtained with standards analysed under the same conditions. The other chlorogenic acids and their derivatives were identified using the same parameters and according to the mass spectra characteristics (*m/z* of the deprotonated molecules and fragmentation patterns of the MS^2 and MS^3 spectra) as compared to those found in the literature [31, 32, 33, 34].

The methods for 5-CQA, caffeine and trigonelline were validated according to the International Conference on the Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use [35] and Horwitz [36].

The validation parameters (selectivity, linearity, limits of detection (LOD) and quantification (LOQ), precision, accuracy and robustness) were determined for the spray dried GCFE. The linearity was investigated over the range from 99.8 to 1742.05 µg mL^{-1} (5-CQA), 39.95 to 474.71 µg mL^{-1} (caffeine) and 94.98 to 452.16 µg mL^{-1} (trigonelline). The LOD and LOQ values were calculated from the relative standard deviation (RSD) and slope (S) of the calibration curve. Selectivity was evaluated comparing the chromatogram of the mobile phase, sample solution, standard and blank (methanol). The spectral similarities of the 5-CQA, caffeine and trigonelline peaks of the standards and samples were also evaluated by comparing the UV spectra in the wavelength range of 262 – 325 nm. The precision was determined at two levels: repeatability (intra-day) and intermediate precision (inter-day). Repeatability was verified in triplicate using three injections of the highest, mean and lowest concentrations of the analytical curve. The intermediate precision was evaluated following the same process, on two different days by different analysts. The accuracy of the method was obtained by a recovery analysis. Three concentration levels of GCFE in triplicate corresponding to 50, 100 and 150%

of the concentrations of the standards in the linear range, with or without the addition of a known amount of a standard solution containing 5-CQA, caffeine and trigonelline. The robustness of the method was determined by comparing the results obtained for the 5-CQA, caffeine, and trigonelline contents with the retention times obtained under the original and modified analytical conditions. Three analytical parameters were selected for variation: oven temperature of 30ºC changed to 29ºC and 31ºC, the aqueous solution of citric acid (pH 2.5) of mobile phase A to formic acid (pH 2.5), and the column batch coupled to another chromatographic system.

2.5 Percolation extraction process

2.5.1 Experimental design

A central composite rotatable design (CCRD) was used to determine the influence of the ethanol concentration in the hydro-ethanolic solvent and the proportion (*w/w*) of material to solvent on the extraction efficiency. A 2^2 CCDR was used, consisting of 4 assays (-1 and +1), 3 central points and 4 axial points (-1.41 and +1.41) carried out in random order with 11 combinations. The extraction process was carried out in three batches, giving a total of 33 experiments (table 1). A second-order polynomial equation (Eq. 1) was fitted to each response to study the effect of the variables and describe the process mathematically.

$$y = a_0 + \sum_{i=1}^{n} a_i x + \sum_{i=1}^{n} \sum_{j=i+1}^{n} a_{ij} x_i x_j + \sum_{i=1}^{n} a_{ii} x_i^2$$

(Eq. 1)

Where y is the predicted response, a_0 , a_i , a_{ii} and a_{ij} are the regression coefficients and x_i, x_j are the coded levels of the independent variables i and j. Model adequacy was evaluated using the F ratio and coefficient of determination (R^2) at a 5% level of significance.

The experimental results were analysed using the Statistic Software version 7.0 where the two-way linear and quadratic interactions were only included for factors with significant effects ($p < 0.05$).

Table 1. Optimization of the extraction of 5-CQA, caffeine and trigonelline from green coffee by CCRD using the independent variables ethanol concentration in the hydro-ethanolic solvent (X_1) and Solid to solvent proportion (X_2)

Variables	-1.41	-1	0	+1	+1.41
ethanol concentration in the hydro-ethanolic solvent (X_1, %)	20	32	50	68	80
Solid to solvent proportion (X_2, w/w)	0.25:10	0.35:10	0.63:10	0.9:10	1:10

2.5.2 Process Yield

The residual organic solvent from each GCFE obtained during the optimization process was removed under vacuum in a rotary evaporator (T 40 ± 2ºC), and the concentrated extract frozen at -80ºC and freeze-dried for 120 h at -92ºC below 60 μmHg. The extract yield was determined as the ratio of the weight of whole green coffee fruit powder applied to the extractive process to the mass of freeze-dried GCFE.

2.6 Large scale preparation of spray dried GCFE

The phytochemical constituents were obtained from whole green coffee fruits (*Coffea canephora*) under the optimized percolation conditions, and the concentrated extracts dried by spray drying to simulate an industrial scale production. Briefly 900 g of plant material was percolated with approximately 10.000 g of ethanol (68% *w/w*), followed by concentration in a rotary evaporator under vacuum (T ≤ 40º C) to obtain a concentrated extract with at least 14% of solids. The concentrated liquid extract showed an ethanol content of 3.7% (*v/v*), relative density of 1.05 g mL^{-1} (20ºC), viscosity of 19.65 mPAs (Rheometro Brookfields® Model DV-III+ pro LV series, at 28 °C) and a residual solid content of 14.2% (*w/w*). The spray dryer conditions were fixed as follows: feed flow rate 0.5 $L.h^{-1}$, drying air flow 45 $L.min^{-1}$, and the inlet and outlet temperatures at 160 ± 2°C and 125 ± 2ºC, respectively.

2.6.1 Morphological characteristics and size distribution of the dried extract

The spray dried GCFE was mounted on the sample holder using carbon tape and then coated with gold using a Denton II Vaccum sputterer and examined using a JEOL®scanning electron microscopy (SEM) model JSM – 6610, equipped with EDS, Thermo Scientífic NSS Spectral Imaging. SEM was carried out at 5kV with magnification of times 1000 and 2500, as

well as at 7Kv with magnification of times 10000. The particle size distribution was measured using Scandium Software and expressed as the mean of six repetitions.

2.6.2 Accelerated stability study

The accelerated stability study was carried out according to the Stability Testing Guideline for Dietary Supplements provided by the NSF International organization [38]. For this, spray-dried GCFE was stored in white polypropylene jars (n=3) for 6 months at 40 °C in an oven with air circulation. Sample stability was monitored according to the water activity, reducing capacity, and the 5-CQA, caffeine and trigonelline contents as determined by HPLC-DAD-MS at five different storage times: T0 (initial time), T1 (after 15 days), T2 (after 45 days), T3 (after 60 days) and T4 (after 180 days). The analyses were carried out in triplicate, the samples for each replicate being taken from different stored polypropylene jars.

To analyse for water activity, a sample from each jar was added to a cartridge inside the hermetic chamber of a thermo hygrometer Testo® 650 (Testo AG, Lenzkirch, Germany). This procedure was carried out at zero time before storage, and after 15, 45, 60 and 180 days of storage.

The reducing capacity of the hydrophilic extract was determined by the Folin–Ciocalteu colorimetric method [39]. The reaction mixture contained the following reagents (final volume = 3000 μL): two concentrations of spray dried GCFE or standards (5-caffeoylquinic acid, caffeine and trigonelline) dissolved in ultrapure water (232 and 309 $\mu g.mL^{-1}$, Folin–Ciocalteau reagent, and sodium carbonate solution (2.3%, w/v). The absorbance was monitored at 765 nm after 120 min at 25 °C, and the components quantified by way of an analytical curve prepared with gallic acid in the range between 37.5 and 100.16 mg. L^{-1}, and the results expressed in milligrams of gallic acid equivalents per gram of extract (mg $GAE.g^{-1}$). Ascorbic acid was used as the positive control (920.8 ± 1.06 mg $GAE.g^{-1}$) [31].

2.7 Data analysis

In the present study, all measurements and analyses were carried out in triplicate. The extraction process was optimized by using a central composite rotational design (CCRD) and the analysis of variance (ANOVA). The results obtained from the CCRD were analysed using the online software Protimiza Experimental Design, the results of the accelerated studies were analysed by one-way ANOVA and the differences between the means by the Tukey-Kramer test using the statistical software STATISTICA 7.0. The data were presented in tables and

graphs as the mean ± standard deviation. Differences were considered statistically significant at $p < 0.05$ and $p < 0.01$.

3. Results and discussion

3.1 Validation of the method to determine 5-CQA, caffeine and trigonelline by HPLC-DAD

The method used in this study was previously validated for roasted coffee beans [32] and coffee brews [34]. However, in the present study a different food matrix was used comprised of whole green coffee fruits, so the method was validated for this matrix, since analytical methods should, at least, be partially revalidated if a change in matrix within a species occurs [39, 40].

Fig 1 shows the chromatograms obtained for the GCFE sample (A), 5-CQA standard (B) and the caffeine and trigonelline standards (C). The peak profiles followed the same pattern reported by Farah et al. [32] and Rodrigues et al. [34] for roasted coffee beans and coffee brews, respectively. The selectivity of the method was observed by comparing the chromatographic profiles of the GCFE sample (Fig 1A), 5-CQA standard (Fig 1B), caffeine and trigonelline standards (Fig 1C) and methanol (Fig 1D), as well as observing the spectral similarity between the 5-CQA, caffeine and trigonelline peaks in the GCFE (Figs 2A, 2C and 2E) and in the standards (Figs 2B, 2D and 2F). Interfering compounds were not observed at the retention times of 5-CQA, caffeine and trigonelline in the sample, and the UV spectrum of the standards was identical to that of the GCFE.

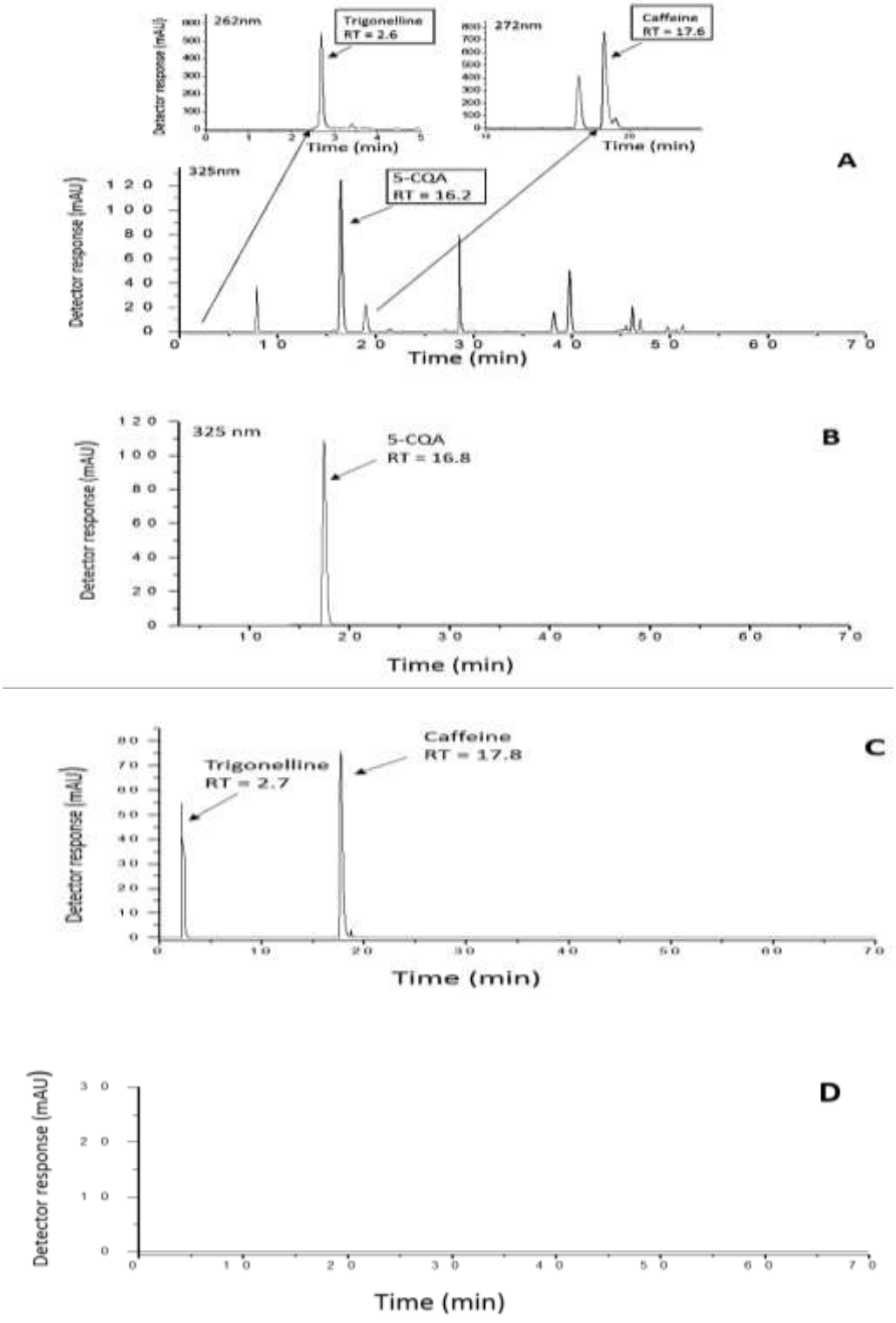

Fig 1. Chromatogram of the GCFE (A); 5-CQA standard (B); caffeine and trigonelline standards (C), and methanol (D) obtained by HPLC-DAD, recorded at 262, 272 and 325 nm. Chromatographic conditions: C18 ODS column (250 x 4.6 mm^2, 5 µm); mobile phase of 80% (v/v) 10 mM citric acid (pH 2.5) and 20% (v/v) of methanol (solvent A) and methanol (solvent B) using a gradient, flow rate of 1.0mL min^{-1}, column temperature of 30ºC; injection volume 20 µL.

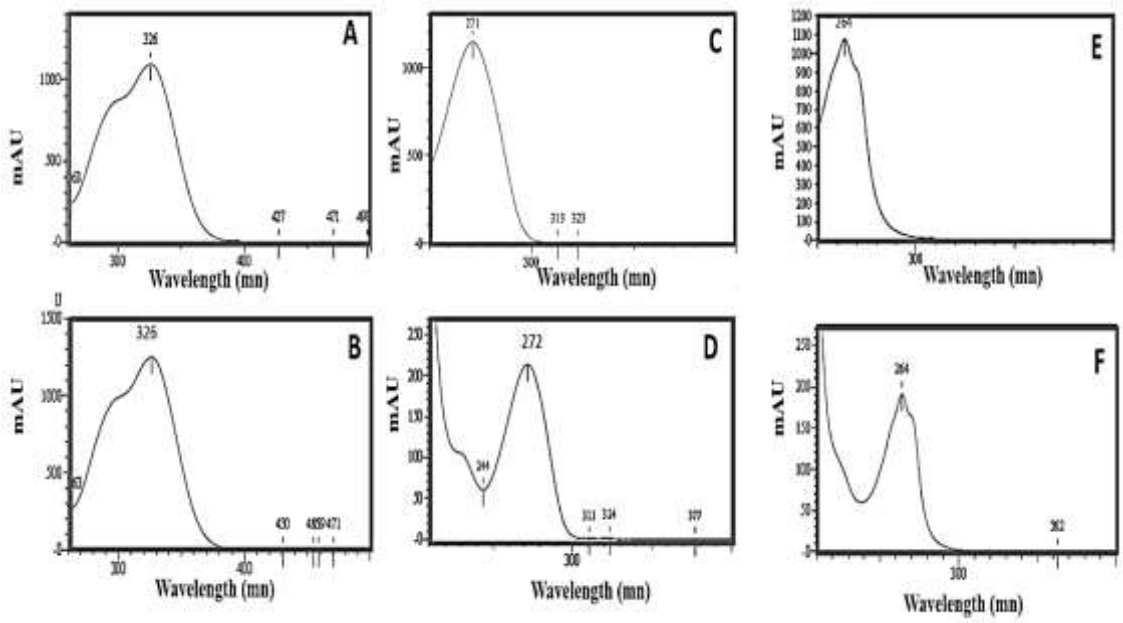

Fig 2. UV spectra of 5-CQA (A), caffeine (C) and trigonelline (E) in GCFE and of the 5-CQA (B), caffeine (D) and trigonelline (F) standards, obtained by HPLC-DAD in the range from 262 to 400 nm.

The separation method was linear within the range studied, showing analytical curves with R^2 above 0.99 ($p < 0.05$) at six concentration levels. Table 2 shows the values obtained for LOQ, LOD and repeatability, calculated from the standard deviation of 3 analyses of the same sample at three different levels and the respective values for recovery.

Table 2. Limits of detection (LOD), limits of quantification (LOQ), repeatability and recovery at three different spike levels for 5-CQA, caffeine and trigonelline in the dried green coffee fruit extract

Compound	LOD ($\mu g.mL^{-1}$)	LOQ ($\mu g.mL^{-1}$)	Spike level ($\mu g.mL^{-1}$)	Repeatability (%)[a]	Recovery (%)[b]
5-CQA	0.98	2.95	295.95	3.76	102.96 (1.06)
			606.03	2.05	101.67 (2.46)
			1220.77	2.55	100.78 (1.66)
Caffeine	7.36	21.04	233.06	3.40	100.17 (1.83)
			433.55	1.89	97.29 (1.91)
			851.68	3.36	99.79 (1.61)
Trigonelline	3.20	9.62	72.95	4.83	95.81 (2.15)
			154.62	4.01	97.63 (0.6)
			320.56	4.19	103.66 (2.31)

5-CQA = 5-caffeoylquinic acid

[a]Repeatability is given as the relative standard deviation (RSD, %).

[b]Relative standard deviation (%) is given in parentheses (n = 3).

The values found for the RSD (%) were less than 5% for all the triplicates of the chemical markers at the low, medium and high concentrations, i.e., for nine determinations, as recommended by ICH [34]. The intermediate precision performance, in which the analysis was run by a different analyst on different days, also had RSD (%) values below 5% for 5-CQA and caffeine. However, for trigonelline, the RSD was 6.12 %. Nevertheless these results are in agreement with Horwitz [36].

Table 3 shows the results obtained in the robustness test for 5-CQA, caffeine, and trigonelline, with changes in temperature, mobile phase and combined changes of the mobile phase and column set-up when coupled to another chromatograph. According to ICH [35] the aim of the robustness test is to show the reliability of the analysis when faced with variations in the method parameters. All the parameters assessed resulted in RSD values below 5%, except for trigonelline (7.41%) when mobile phase, column batch nº and chromatograph were changed simultaneously. However, the retention times had RSD below 5% for caffeine, trigonelline and 5-CQA using different mobile phases, column set-ups and chromatographs concurrently. These results demonstrate the level of confidence of the method when faced with small and deliberate variations in the analytical parameters. The demonstration of robustness is critical for the transference of the analytical method parameters to other laboratories [41, 42].

3.2 Percolation extraction process

Three batches of whole green coffee fruit powder were applied at each run point of the CCDR experiment. The yield of the extractive process ranged from 16 to 27% on a dry weight basis and the 5-CQA content ranged from 521 to 9751 $g.100g^{-1}$ on a dry weight basis under different conditions. The results obtained under the same extraction conditions showed no significant difference between batches for yield or for the 5-CQA content according to ANOVA ($p<0.05$) and the Tukey method of multiple comparisons, indicating that the process used to obtain the standardized extract was reproducible.

Table 4 shows the results obtained for the 5-CQA content and extractive process yield for three batches of the 11 different experiments generated by the CCRD. Multiple linear regression was carried out using the quadratic polynomial model (Eq. 1) based on the results shown in table 4. The quadratic model for the 5-CQA ($p = 0.0035$) content and extraction yield ($p = 0.0412$) were significant. In addition the R^2 value of 98.6% obtained for the 5-CQA content confirmed the model adequacy. However the models obtained for the extraction yields of the trigonelline and caffeine contents were not adequate, showing R^2 values of 41, 32 and 13%,

respectively. Table 5 shows the results obtained in the analysis of variance (ANOVA) of 5-CQA.

The data in table 5 show significant linear (p=0.0000) and quadratic (p=0.0000) effects for the ethanol concentration in the range from 65 – 80%, and a quadratic effect for the solid to solvent proportion (p=0.0444), as well as for the interaction between the solid to solvent proportion and the ethanol concentration (p=0.000057) in the range from 0.8:10 – 1:10 (w/w) and 65 – 80% respectively, indicating an influence of the 5-CQA content. However, the response surface for the 5-CQA content (figure 3) did not allow for the determination of the optimal extraction conditions, although showing a significant tendency around the variables studied. For the extraction yield, a significant positive linear effect (p= 0.0013) of the ethanol concentration in the range from 65 – 80%, and a quadratic effect (p= 0.055) of the solid to solvent proportion in the range from 0.8:10 – 1:10 (w/w) were observed.

Table 3. Results of the robustness test for the quantification of trigonelline, caffeine and 5-CQA in the GCFE by an analytical HPLC method

Parameter	5-CQA Average (RSD% intra)		Caffeine Average (RSD% intra)		Trigonelline Average (RSD% intra)	
	Rt	Area	Rt	Area	Rt	Area
Column oven temperature (ºC)						
29	17.59 (0.20)	32723432 (0.10)	19.43 (0.13)	23860510 (2.43)	2.92 (0.19)	3733033 (0.72)
30	17.09 (0.24)	34371095 (2.04)	18.98 (0.40)	23607313 (1.52)	2.92 (0.00)	3780450 (1.94)
31	17.10 (1.53)	33238176 (2.41)	18.93 (1.09)	22382846 (1.06)	2.92 (0.20)	3763492 (1.03)
RSD% inter	1.62	2.70	1.36	3.32	0.17	1.29
Mobile phase						
Citric acid (10 mM) (pH 2.5)	17.09 (0.24)	34371095 (2.05)	18.98 (0.40)	23607313 (1.52)	2.92 (0.00)	3780450 (1.94)
Formic acid (0.3%) (pH 2.5)	17.47 (0.33)	34736737 (2.08)	19.31 (0.44)	23645321 (2.10)	2.90 (0.20)	3818593 (2.72)
RSD% inter	1.21	1.93	1.01	1.64	0.33	2.19
Column batch nº, mobile phase and chromatograph						
A	17.09 (0.24)	34371095 (2.05)	18.98 (0.40)	23607313 (1.52)	2.92 (0.00)	3780450 (1.94)
B	16.32 (0.14)	32854821 (1.43)	18.35 (1.63)	23872574.67 (2.86)	2.68 (0.00)	4319276 (1.13)
RSD% inter	2.55	2.94	2.12	2.15	4.69	7.41

RSD (%) = Relative Standard Deviation; Rt = Retention Time; 5-CQA = 5-caffeoylquinic acid; GCFE = dried green coffee fruit extract. A= Column batch nº 4258319 Shim-pack CLC-ODS (M); (Shimadzu HPLC, Kyoto, Japan) equipped with a binary pump (LC-10AD); 10 mM citric acid (pH 2.5). B = Column batch nº 4258432 Shim-pack CLC-ODS (M); (Shimadzu HPLC, Kyoto, Japan) equipped with quaternary pump (LC-20AD); 0.3% formic acid (pH = 2.5).

Table 4. Central composite rotatable design (CCRD) for the percolation parameters (independent variables, X_1 and X_2) and the response for the 5-CQA, caffeine and trigonelline contents and extraction yields (dependent variables)

Assay	**X_1 (%)**	**X_2 (*w/w*)**	**Yield (%)**	**5-CQA (mg.100g^{-1})**	**Caffeine (mg.100g^{-1})**	**Trigonelline (mg.100g^{-1})**
1	32	0.35:10	21.3±0.6	1932±106^{a}	7803±347^{aA}	2993±95^{abB}
2	68	0.35:10	24.1±1.5	6026±207^{A}	7924±484^{aA}	2934±162^{abB}
3	32	0.9:10	16.2±0.4	3570± 150^{b}	8078±659^{a}	3030±26^{bB}
4	68	0.9:10	25.0±1.2	9751±274^{B}	6822±127^{aA}	2664±118^{ab}
5	20	0.63:10	18.5±0.5	521±60^{c}	7497±286^{aA}	2957±141^{aBb}
6	80	0.63:10	20.8±0.1	8354±298^{C}	8002±355^{a}	3054±90^{B}
7	50	0.25:10	18.8±2.0	4173±383^{b}	6642±652^{A}	2409±93^{A}
8	50	1:10	19.9±1.7	7838±572^{C}	7119±699^{aA}	2646±187^{aA}
9	50	0.63:10	26.9±1.6	6282±451 ^{A}	7596±277^{aA}	2758±181^{aAbB}
10	50	0.63:10	22.2±0.8	6321±496^{A}	7145±158^{aA}	2526±109^{A}
11	50	0.63:10	21.8±1.2	6400±455^{A}	7732±359^{aA}	2979±118^{aAb}

X_1 – Ethanol; X_2 – Solid-to-solvent proportion (w/w). The results are expressed as the mean ± standard deviation. Means with the same letters in the same column do not differ significantly from one another according to Tukey's test with a 5% significance level.

Table 5. Analysis of variance (ANOVA) of the 5-CQA content (mg.100g^{-1}) for the quadratic polynomial regression model

Factor	Sum of square	Degrees of freedom	Mean squares	F value	P
X_1	173299389	1	173299389	1520,751	0.000000**
X_1^2	14093343	1	14093343	123,673	0.000000**
X_2	39816804	1	39816804	349,404	0.086804
X_2^2	506219	1	506219	4,442	0.044486*
X_1. X_2	2587431	1	2587431	22,705	0.000057**
Lack of fit	213992.7	1	213992.7	14.7	0.06419
Pure error	3076824	27	113956	1772.452	
Total sum of squares	233981236	32			

X_1 – Ethanol; X_2 – Solid to solvent proportion
* $p < 0.05$
** $p < 0.01$

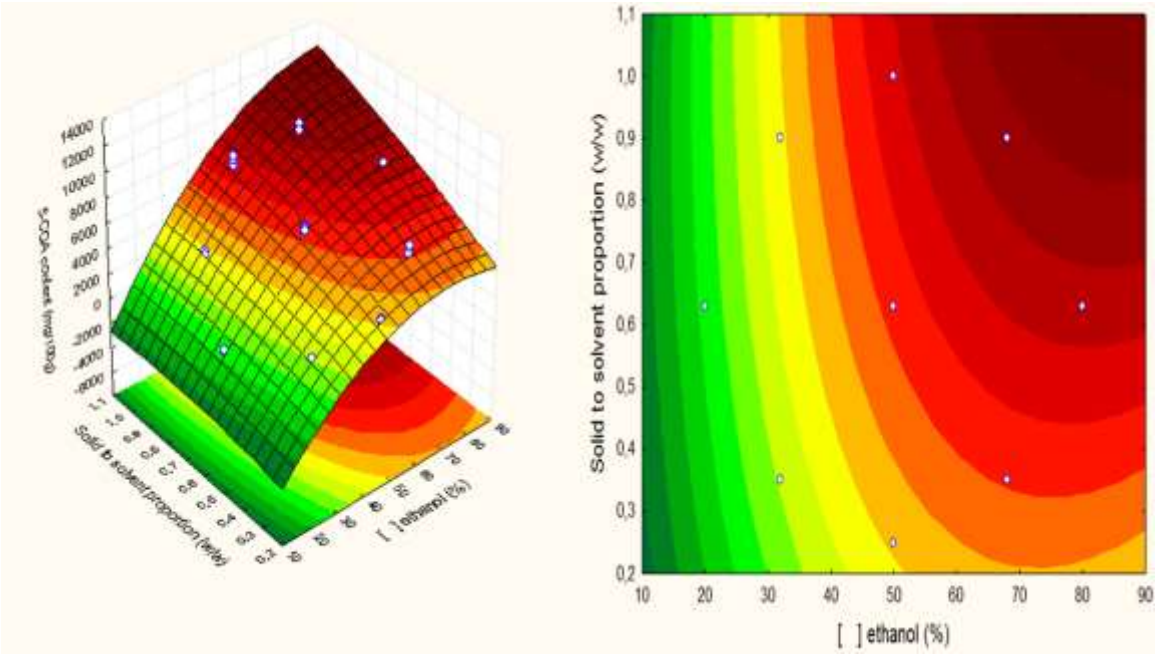

Fig 3. Three-dimensional (A) and bi-dimensional (B) response surfaces showing the effect of the ethanol concentration in the extraction solvent and the solid to solvent proportion on the 5-CQA content

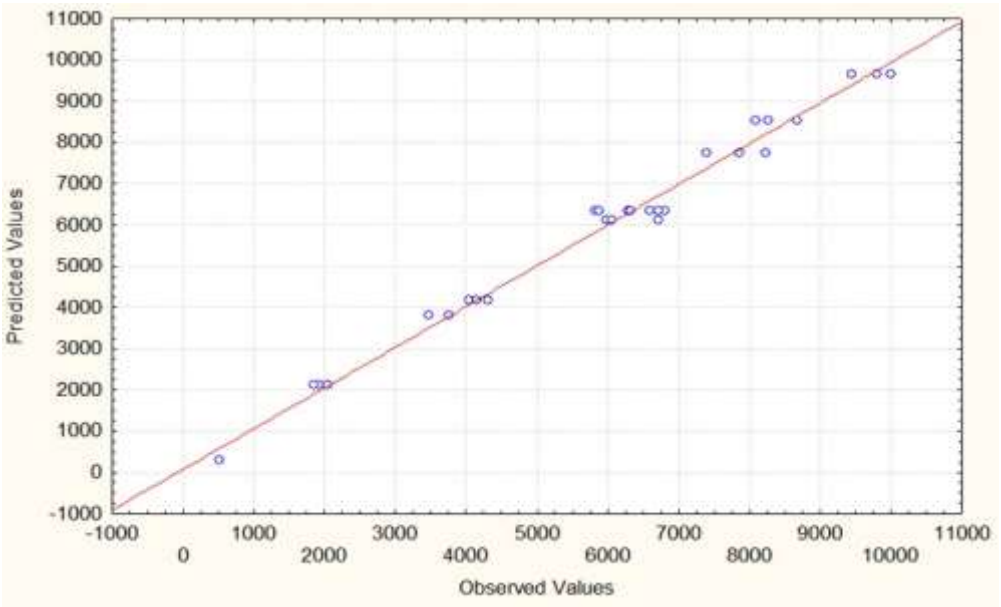

Fig 4. Correlation between the observed values (obtained from the experimental analysis) versus the predicted values (calculated using the statistical model) for the 5-CQA content

Figure 4 shows the correlation between the data predicted by the model versus the observed data, showing high correlation between them.

The parameter solid to solvent proportion (X_1) analysed for the 5-CQA content and the yield response surface graph indicated that the maximum point was outside the experimental area. In these cases, in order to obtain the optimal value, increased levels should be used in a new experiment. However, in this case it was not feasible to further increase the solid to solvent proportion due to the high intumescence of the vegetable material as a result of the presence of mucilage from the coffee fruit pulp.

The ethanol concentration in the hydroalcoholic solvent (X_2) was a parameter assessed since ethanol is the solvent most commonly used in the commercial extraction process of chlorogenic acids [43] and, since it is non-toxic, it adheres to the green chemistry principles. In addition, 100% ethanol was not assessed in this study, since water improves the solubility when present at a level of at least 20% in the solvent mixture [43]. According to Dibert & Cros [44] for the extraction of chlorogenic acid, a binary mixture of an alcohol and water is required. As reported by Upadhyay et al. [24] pure ethanol was less effective in the extraction of chlorogenic acids from green coffee beans by microwave-assisted extraction than pure water. This could be explained by the high dipole-moment of the water molecules, which help the matrix to absorb microwave energy [45]. To the contrary, by maceration, pure water is less effective in the extraction than the mixture of ethanol in water, due to the high solubility of polyphenols in hydroalcoholic solutions [46].

The three-dimensional response surface presented in figure 3 (A) for the independent variables of ethanol concentration (%) and solid to solvent proportion (w/w) indicated changes in the 5-CQA content under different percolation conditions. When the ethanol concentration increased from 32 to 68% for the same solid to solvent proportion, the 5-CQA content increased from 570 to 9751 $mg.100g^{-1}$. In addition, when the solid to solvent proportion decreased from 0.9:10 (w/w) to 0.35:10 (w/w) for the same ethanol concentration (68%), the 5-CQA content decreased from 9751 to 6026 $mg.100g^{-1}$.

Caffeine and trigonelline are members of the class of methylxanthine alkaloids. These compounds can be found homogeneously distributed in vegetable matrixes complexed with chlorogenic acids, and both are highly soluble in water and ethanol. Methylxanthines are more efficiently extracted by water due to the greater number of hydrogen atoms available than in ethanol [47]. However in the present study, only the quadratic effect ($p=0.0006$) of the ethanol concentration (%) showed significance for the trigonelline content, and the interaction between the linear effect of the ethanol concentration and the solid to solvent proportion ($p=0.0445$) for the caffeine content, since under different extraction conditions, the caffeine and trigonelline

contents varied from 6642 to 8078 and from 2409 to 3054 mg.100g^{-1}, respectively. Smaller caffeine and trigonelline contents ($p<0.05$) were found when the solid to solvent proportion decreased.

Various methods using traditional and non-traditional procedures have been assessed for the extraction of chlorogenic acids and its derivatives and of caffeine from green coffee, and extracted smaller amounts than the percolation method presented in the present study. For example, Lekar et al. [1] extracted about 5300 mg.100g^{-1} GCA using a subcritical water medium and Upadhyay et al. [24] obtained levels of chlorogenic acids and caffeine in the range of from 4700 to 8400 mg.100g^{-1} and from 3440 to 7250 mg.100g^{-1}, respectively, using microwave-assisted extraction. The extraction of chlorogenic acids and caffeine using pure supercritical CO_2 or supercritical CO_2 modified with 5% ethanol (w/w) or 5% isopropyl alcohol (w/w) was efficient in extracting caffeine, obtaining 1.7, 17 and 2 g, respectively, of caffeine/g of solvent, but chlorogenic acids were only present as traces in all the extracts [47]. Recent patents on the application of different methods for the extraction of bioactive compounds from cherry coffee achieved chlorogenic acid contents in the range from 6740 to 10000 mg.100g^{-1}, considering the sum of the chlorogenic acid derivatives 3-CQA, 4- CQA and 5-CQA [48, 49], while in the present study, the 5-CQA content alone was around 9751 mg.100g^{-1}.

3.3 Obtaining the spray dried GCFE

Spray dryer are traditionally used in the herbal processing industries to obtain dry powders, granulates or agglomerates by reducing the water activity to the low level required to stop bacterial degradation. In general, the products obtained by spray drying are more soluble, concentrated and stable [50].

Considering the instrumental parameters applied in the spray drying process, the hydroalcoholic extract obtained in experimental run number 4, i.e., 68% of ethanol in the hydro-ethanolic solvent and a solid to solvent proportion equal to 0.9:10 (w/w), was efficiently atomized to produce a fine dry powder with a low water activity equal to 0.12, which is below the range required for bacterial growth. The production yield of spray dried GCFE was 26 ± 2% (*w/w*), close to that obtained by the freeze drying technique, but still below the optimal yield considered by Bandhari et al. [51] and by Best@buchi [52] for lab-scale spray dryers of in the 50–70% range. However, in the spray drying technique, the processing yields of herbal extracts have been shown to be variable and totally dependent on the characteristics of the feed liquid (viscosity, solids content, flow rate). In this context, some authors recommend the use of

different drying adjuvants, since, when no carrier aids are used, significant amounts of wall deposits can occur, decreasing the process yield [51, 53]. Generally, sticking to the spray dryer walls is caused by the presence of low-molecular weight sugars in the herbal extracts, due to their low glass transition temperatures [51, 54]. The presence of phenolic acids in the chemical compositions of the extracts appears to be involved in decreasing extract stickiness. Thus Medina-Torres et al. [55] dried a plant extract with and without gallic acid, since these authors reported that the powder containing gallic acid had a much higher glass transition temperature than the powder without the phenolic acid content. Considering that in the present study the GCFE was dried without the use of any adjuvants, the recovery achieved was efficient, showing similar yields to those described in the literature for plant extract powders [53, 56].

3.3.1 Identification of xanthines and CGA derivatives

The mass spectrometry (MS) chromatographic method with an electrospray ionization (ESI) interface was used to identify the chemical compounds present in the spray dried GCFE. Under these ESI conditions, trigonelline, caffeine and theophylline could effectively be transformed into abundant molecular ions $[M + H]^+$ *m/z* 138, 181 and 195, respectively. Twenty one hydroxycinnamic molecular ions $[M – H]^-$ *m/z* ranging from 179 to 520 were also identified, and the CGA isomers were discriminated using the combined data of the following parameters: elution order in the reverse phase, chromatography of the standards, UV–visible and mass spectra characteristics with their MS^2 and MS^3 fragment patterns and their relative intensities, in accordance with the hierarchical scheme described by Clifford et al. [33], Rodrigues et al. [31] and Rodrigues et al. [34].

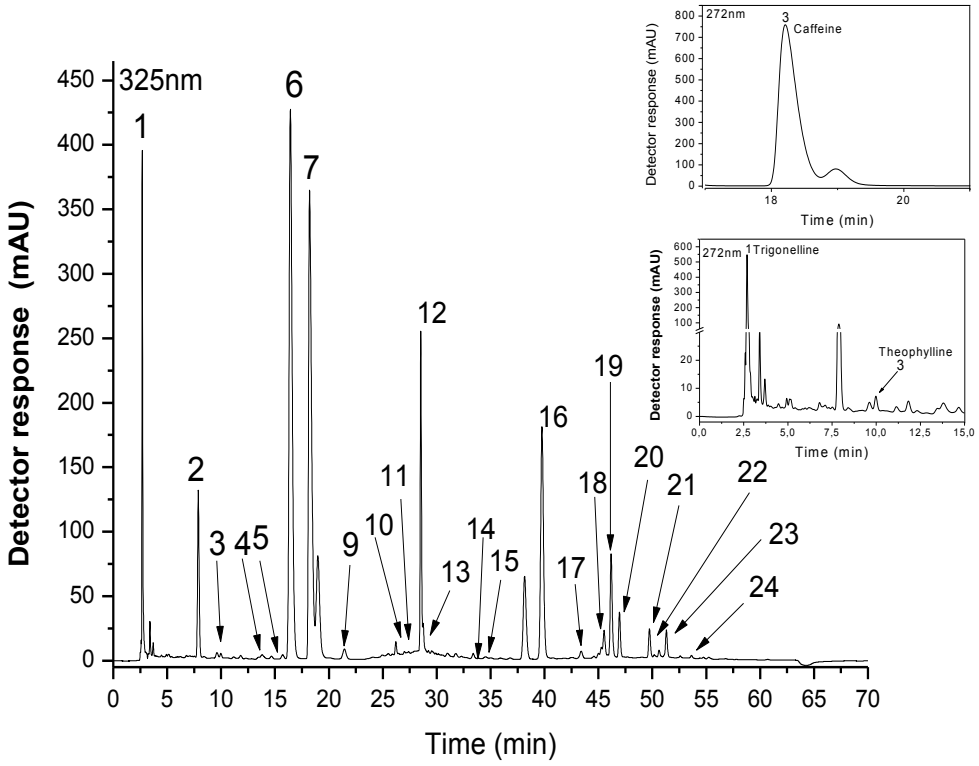

Fig 5. Chromatographic profile of the GCFE obtained by HPCL-DAD-MS, recorded at 325 and 272 nm. Table 6 shows the compound identification.

Table 6 shows all the data for the hydroxycinnamic acids and xanthines using the recommended IUPAC numbering system [57] and Fig. 5 shows a typical chromatogram obtained for green coffee at 325 and 272 nm.

According to Rodrigues et al. [34] the hydroxycinnamic acids are subdivided into 4 groups: CGA, chlorogenic acid lactones, cinnamoyl-amino acid conjugates and free cinnamic acids. The total of 22 hydroxycinnamic derivatives found in the GCFE were identified as 17 CGA, 3 cinnamoyl-amino acid conjugates, 1 free cinnamic acid and 1 chlorogenic acid lactone. Of the CGA lactone group, only 3-CQL (*m/z* 335) was detected. Normally, the lactones are formed during roasting to less than 10% moisture content, when a water molecule from the quinic acid moiety is removed forming an intramolecular ester bond [58]; thus during drying (160 °C) of the concentrated GCFE, 3-CQL could be formed. On the other hand, Rodrigues et al. [31] observed no lactones in green coffee samples, but small quantities of 3-FQL were found by Perrone et al. [59] in the Mundo Novo cultivar and 4-FQL and 3,4 diCQL were found by Farah et al. [32] in green Robusta beans.

CGA has been reported as the major group in green coffee, 5-CQA being the predominant form, followed by 4-CQA and 3-CQA [58, 59], and these compounds were identified at *m/z* 353. These 3 isomers were distinguished by the MS^2 and MS^3 fragmentation pattern and relative intensity of the MS^2 and MS^3 ions. The 3-CQA (peak 2) and 5-CQA (peak 6) showed the same MS^2 base peak at *m/z* 191([quinic acid H]$^-$) but could be differentiated by the relative intensity of the secondary ion from the caffeoyl moiety ([caffeic acid H]$^-$) at *m/z* 179, as reported previously by Clifford et al. [33] and Rodrigues et al. [34].

3.4 Morphological characteristics and size distribution of the dried extract

Fig. 6 shows the SEM images obtained for the spray dried GCFE with magnification levels of x1, 000, x2, 500 and x10,000. The powdered extract particles were mostly spherical with a smooth surface, but some depressions and wrinkles were also present. The mucilage present in the pulp of the green coffee fruit samples could have been responsible for forming a smooth surface, even without the addition of drying aids. In general, the great amount of wrinkles on the particle surface is responsible for powder agglomeration and, consequently, for poor powder flowability [50]. The particle diameters were of homogeneous size with a unimodal distribution, but despite the homogeneity, different sizes were found in the spray dried GCFE, within a range from 0.72 – 5.12 µm (Fig 6). The particle size is expressed in terms of the particle diameter, with a greater frequency of between 0.72 and 2.0 µm. According to Gong et al. [50], spray dried powders often have a small particle size (<50 µm). Smaller particles have a greater surface area and therefore better solubility [60], good solubility being an essential characteristic of food ingredients, since foods contain a lot of water.

Table 6. Chromatographic and mass spectroscopic characteristics of the CGA and derivatives and xanthines identified in the spray dried GCFE as obtained by HPLC-DAD-MS.

Peak	T_R (min)	Compound	Abbreviation	λ_{max} (nm)	$[M - H]^-$ (*m/z*)	$[M + H]^+$ (*m/z*)	Fragments (*m/z*)
1	2.9	Trigonelline	Trig	265		138	MS^2 [138]: **121**, 110, 142, 94
2	8.0	3-caffeoylquinic acid	3-CQA	239, 326	353		MS^2 [353]: **191**, 179, 135, 173 MS^3 [353 → 191]: **127**, 93, 173, 111, 81
3	11.6	Theophylline	Theoph	274		181	MS^2 [181]: **163**, 190, 123, 137
4	12.6	3-*p*-coumaroylquinic acid	3-*p*CoQA	226, 313	337		MS^2 [337]: **163**, 119 MS^3[337→163]: 119
5	15.5	3- Feruloylquinic acid	3-FQA	236, 324	367		MS^2 [367]: **193**, 133, 173 MS^3 [367 → 193]: **133**, 149
6	16.2	5-caffeoylquinic acid	5-CQA	238, 326	353		MS^2 [353]: **191**, 179 MS^3 [353 → 191]: **127**, 173, 93, 111, 144, 85
7	18.7	4-caffeoylquinic acid	4-CQA	239, 326	353		MS^2 [353]: **173**, 179, 191, 135 MS^3 [353 → 173]: **93**, 111, 155, 71
8	19.8	Caffeine	Caffein	274		195	MS^2 [195]: **163**, 137, 109
9	21.5	Caffeic acid	CA	236, 322	179		MS^2 [179]: **135,** 119
10	27.1	5-*p*-Coumaroylquinic acid	5-pCoQA	234, 312	337		MS^2 [337]: **191**, 163, 173 MS^3[337→191]: 127, 85
11	27.8	3-Caffeoyl-1,5-lactone	3-CQL	240, 326	335		MS^2 [337]: **161**, 135, 191, 173
12	28.7	5-Feruloylquinic acid	5-FQA	238, 326	367		MS^2 [367]: **191,** 173 MS^3 [367 → 191]: **127**, 173, 85

13	28.8	4-Feruloylquinic acid	4-FQA	238, 236	367	MS^2 [367]: **173,** 191 MS^3 [367 → 173]: **93,** 155, 111
14	33.3	Caffeoyltyrosine	CTyr		342	MS^2 [342]: **206,** 163, 135 MS^3[342→206]: **163**, 119
15	34.6	3,4-Dicaffeoylquinic acid	3,4-DiCQA	242, 325	515	MS^2 [515]: **353,** , 173, 335, 179, 191 MS^3 [515 → 353]: **173**, 179, 191, 135
16	39.4	3,5-Dicaffeoylquinic acid	3,5-DiCQA	323	515	MS^2 [515]: **353,** 191 MS^3 [515 → 353]: **191**, 179, 135, 173
17	44.8	3-*p*-coumaroyl-4-caffeoylquinic acid	3*p*Co, 4-CQA		499	MS^2 [499]: **353,** 335, 337, 173, 179, 203 MS^3 [499 → 353]: **173**, 179, 191, 135
18	45.1	3-Feruloyil, 4-caffeoylquinic acid	3F, 4CQA	242, 328	529	MS^2 [529]: **353**, 367, 335, 349, 173 MS^3 [529 → 353]: **173**, 179, 191, 135
19	45.5	3-Cafeoylquinic, 4-Feruloylquinic acid	3C, 4FQA	240, 323	529	MS^2 [529]: **367,** 173, 335, 193 MS^3 [529 → 367]: **173**, 193
20	46.4	4,5 Dicafeoylquinic acid	4,5-DiCQA	244, 328	515	MS^2 [515]: **353,** 203, 173, 299, 255 MS^3 [515 → 353]: **173**, 179, 191, 135
21	49.6	Caffeoyltryptophan	CTrp	290, 322	365	MS^2 [365]:**229,** 186, 135 MS^3 [365 → 229]: **185**, 100, 142
22	51.1	4-Feruloyil, 5-caffeoylquinic acid	4F, 5CQA	241, 328	529	MS^2 [529]: **367,** 173, 193 MS^3 [529 → 367]: **173**, 193, 134
23	51.5	4-Cafeoyl, 5-Feruloylquinic acid	4C,5FQA	241, 326	529	MS^2 [529]: **353**, 367, 173, 191 MS^3 [529 → 353]: **173**, 179, 191, 135
24	53.6	*p*-coumaroyltryptophan	*P*-CoTrp	291, 306	349	MS^2 [349]: **229**, 185 MS^3 [349 → 229]: **185**, 100,142

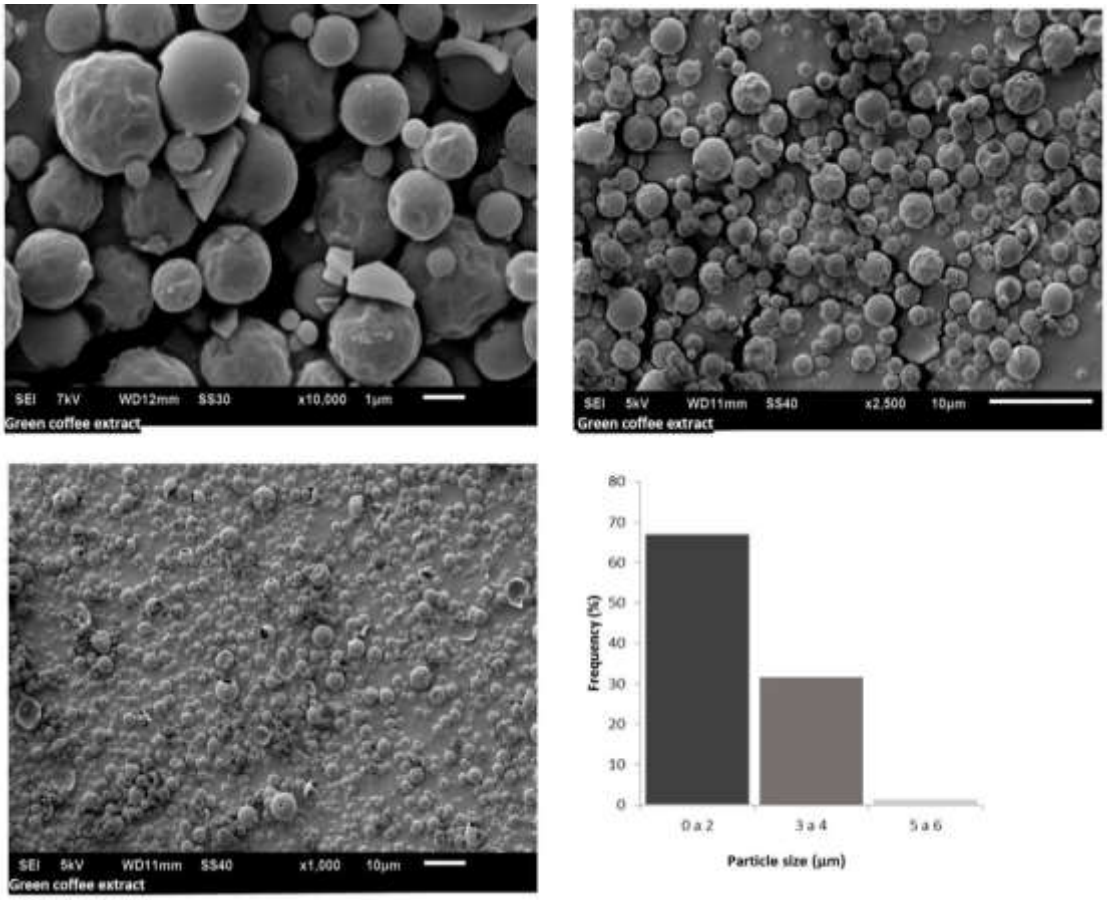

Fig 6. Surface photomicrography of the GCFE by scanning electron microscopy (10,000 x 7kV; 2,500 and 1,000 x 5 kV), and the particle size frequency of the green coffee extract as determined using Scandium® software

3.5 Accelerated stability study

Accelerated shelf-life studies may provide an early indication of long term effects under non-accelerated conditions. The International Conference on the Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use requests that accelerated studies be carried out for three to six months [61]. During this time the samples must be analysed several times to provide data to plot the trends of the results. The recommended storage conditions and tolerances for accelerated stability studies are 40ºC +/- 2ºC and 75% relative humidity (RH) +/- 5% RH, these being the conditions indicated for products with no special storage conditions specified on the label [35, 61]. In the present assay, only the temperature was controlled since the GCFE was stored in closed packaging.

The present shelf-life study of the dietary supplements followed the recommendations of the Stability Testing Guideline for Dietary Supplements provided by the NSF Stability Testing

Working Group [35]. In order to analyse the stability of the powdered products as recommended by the NSF, the water activity and the degradation of the ingredients or bioactive compounds were determined and also the *in vitro* reducing capacity in order to monitor the stability of the spray dried GCFE.

3.5.1 Trigonelline, caffeine and 5-CQA contents in spray dried GCFE

Table 7 shows the caffeine, trigonelline and 5-CQA contents determined in the spray dried GCFE at zero storage time and after 15, 45, 60 and 180 days of storage in an air circulation incubator at 40°C. The 5-CQA concentrations did not vary significantly during the first 60 days of storage at 40ºC ($p > 0.05$), but showed a significant ($p < 0.05$) difference after 180 days. The trigonelline and caffeine contents remained constant for 45 days, but reduced by 7.92 and 4.38%, respectively ($p < 0.05$) after 180 days of storage. These results indicate that, despite the progressive increase in water activity ($p < 0.05$) of the GCFE, which could culminate in the degradation of bioactive compounds, the bioactive markers were relatively stable in the GCFE, with only slight degradation after180 days of storage at 40ºC.

In contrast, the reducing capacity decreased significantly ($p < 0.05$) during the 180 days the stability was monitored. This change in reducing capacity was apparently not connected to the contents of the markers, since the reduction in the chemicals quantified did not exceed 7.92%, whereas the decrease in reducing capacity of the CGE was greater than 34%.

Despite the fact that the caffeine metabolites, especially 1-methylxantine and 1 methylurate, exhibit *in vitro* antioxidant activity [58], the decaffeination process did not apparently influence the reducing capacity in the Folin-Ciocalteau assay [62] to a significant degree. On the other hand, the reducing capacity showed strong correlation with the CGA content [2].

Table 7. The stability of spray dried GCFE during storage for 0, 15, 45, 60 and 180 days at 40ºC as determined by monitoring the trigonelline, caffeine and 5-CQA contents, reducing capacity and water activity

Days	Parameters monitored				
	5-CQA[1] ($mg.100g^{-1}$)	Trigonelline ($mg.100g^{-1}$)	Caffeine ($mg.100g^{-1}$)	Reducing capacity (mg $GAE.g^{-1}$)[2]	Water activity (aw)
0	13174 ± 223^{a}	3470 ± 42^{a}	8805 ± 155^{a}	551 ± 18^{a}	0.121 ± 0.007^{a}
15	13026 ± 97^{a}	3418 ± 35^{a}	8650 ± 23^{a}	539 ± 14^{ab}	0.203 ± 0.003^{b}
45	12859 ± 74^{a}	3489 ± 47^{a}	8688 ± 93^{a}	483 ± 28^{bc}	0.284 ± 0.007^{c}
60	12757 ± 135^{a}	3290 ± 33^{b}	8546 ± 28^{ab}	427 ± 28^{c}	0.331 ± 0.009^{d}
180	12299 ± 241^{b}	3213 ± 107^{b}	8307 ± 217^{b}	359 ± 22^{d}	0.354 ± 0.009^{e}

The values were represented as the mean of three independent experiments ± S.D.
Different letters in the same column mean significant differences ($P < 0.05$)
[1]5-caffeoylquinic acid
[2]The reducing capacity results are expressed as mg gallic acid equivalents/g of sample

In relation to the standards, the spray dried GCFE showed reducing capacities that were about 95 and 64 times higher for trigonelline (5.80 ± 0.2 0.36 mg $GAE.g^{-1}$) and caffeine (8.61 ± 0.36 mg $GAE.g^{-1}$), respectively, but on average 4 and 5 times smaller for ascorbic acid (positive control) and 5-CQA. The results showed that the phenolic acids were the main free radical scavenger compounds present in the GCFE. However, it is well known that the Folin-Ciocalteau reagent reacts with other compounds besides phenols, such as minerals, vitamins, several amino acids and sugars [63]. Considering that extraction by percolation provides a crude extract containing a variety of different substances, since several compounds are carried by the solvent when it passes through the green coffee powder, the decrease in the reducing capacity of the GCFE could be related to the degradation of other chemical compounds besides phenolic acids.

During storage at 40ºC, the decrease in reducing capacity monitored by the Folin-Ciocalteau method may also be attributed to the binding of polyphenols to other compounds (proteins) or to alterations in the chemical structure of the polyphenols, which cannot be extracted or determined by the available methods [64].

Considering that the water activity (aw) is an important parameter, which is linked to the microbiological and chemical stability, the aw of the GCFE was also monitored during the 180

days of storage. The water activity is a determining factor, and aw values lower than 0.5 do not permit the growth of microorganisms [65]. As can be seen in table 8, in the present study the spray dried GCFE showed aw values ranging from 0.12 (zero day) to 0.35 (180 days). Despite the low aw values throughout the storage time, the water activity increased significantly ($p < 0.05$) after 180 days, but nevertheless the range of aw values found during the analysis was favourable to the physicochemical stability of the extract.

4. Conclusion

The results revealed that percolation was an effective method for extracting caffeine, trigonelline and 5-CQA from whole green coffee fruits. Percolation is a good industrial tool showing low operational costs and high process yields, and in the present study greater chlorogenic acid and caffeine contents were extracted than in other studies or using other patents. According to the results of the central composite rotatable design and response surface, the best conditions for the extraction of the bioactive compounds by percolation were an ethanol concentration in the range from 65 – 80 % (w/w) and a solid to solvent proportion in the range from0.8:10 – 1:10 (w/w).

Thus by establishing the extraction parameters, a GCFE was successfully obtained on a large scale by spray drying, showing a reproducible yield and bioactive compound content as compared to the extract obtained on a small scale. Moreover, the GCFE showed good stability throughout 180 days of storage, suggesting its potential application in the pharmaceutical, dietary (sport nutrition supplement, functional food, food additive) and cosmetic industries.

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Supplementary table

Table S1. Analytical HPLC method to determine the repeatability (intra-day) and intermediate (inter-day) precision for the quantification of 5-CQA, caffeine and trigonelline in the GCFE.

Concentration levels	5-CQA			Caffeine			Trigonelline		
	GCFE concentration ($mg.mL^{-1}$)	Area (µAUs)	Content ($\mu g.mg^{-1}$)	GCFE concentration ($mg.mL^{-1}$)	Area (µAUs)	Content ($\mu g.mg^{-1}$)	GCFE concentration ($mg.mL^{-1}$)	Area (µAUs)	Content ($\mu g.mg^{-1}$)
Intra-day (day 1, analyst 1)									
Low	2.65	18351354	126.42	2.65	12612022	93.54	2.65	2163224	34.85
	2.65	17593113	120.33	2.65	12172422	90.02	2.65	1953845	31.24
	2.76	17980902	118.41	2.76	12402655	88.12	2.76	1984933	30.51
Medium	5.18	35027458	130.63	5.18	23879606	91.77	5.18	3822624	32.19
	5.13	33628440	126.31	5.13	23199460	89.99	5.13	3695547	31.39
	5.21	34457380	127.56	5.21	23742874	90.62	5.21	3823180	31.98
High	10.05	64707455	127.77	10.05	45441919	90.57	10.05	7554403	33.22
	10.38	67259127	128.81	10.38	47535001	91.81	10.38	7762822	33.08
	10.46	66425994	126.13	10.46	46923893	89.88	10.46	7709606	32.58
RSD(%)			3,13			1.68			3.98
Intra-day (day 2, analyst 2)									
Low	2.57	17095294	120.34	2.57	11835240	90.35	2.57	1642839	26.85
	2.56	16059760	112.30	2.56	11118618	84.88	2.56	1777252	29.25

	2.56	16179977	113.48	2.56	11193749	85.64	2.56	1771623	29.21
Medium	5.18	31154789	115.20	5.18	21538314	82.56	5.18	3454784	28.98
	5.10	31747440	119.50	5.10	21910975	85.41	5.10	3510908	29.95
	5.12	31835333	119.27	5.12	21974326	85.25	5.12	3690474	31.38
High	10.13	62593610	122.55	10.13	43122189	85.28	10.13	7020934	30.61
	10.10	62072097	121.86	10.10	43356702	86.00	10.10	7112446	31.11
	10.18	60084444	116.90	10.18	41071818	80.79	10.18	7624519	33.12
RSD (%)			3.09			3.04			5.42
Inter-day (Intermediate precision)									
RSD (%)			4.49			4.02			6.12

RSD (%) = Relative Standard Deviation.

CAPÍTULO IV

Microencapsulated green coffee fruit extract obtained by spray-drying: development, standardization and stability study

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ABSTRACT

This research explored the spray-drying microencapsulation of a green coffee fruit extract (GCFE) with a high level of chlorogenic acids, standardized for 5-*O*-caffeoylquinic acid (5-CQA). The optimal conditions for microencapsulation by spray drying were selected using response surface methodology (RSM) and a central composite rotational design (CCRD) to evaluate the effects of replacing part of the gum Arabic (GA) by maltodextrin (MD) as the carrier material and of the ratio of concentrated extract: carrier agent dispersion (w/w). These effects of the independent variables were evaluated on the encapsulation efficiency, process yield, 5-CQA retention, surface morphology, particle size distribution and GCFE stability at 40ºC, as monitored by the water activity (Aw), reducing capacity and the contents of the bioactive compounds 5-CQA, caffeine, trigonelline, caffeic acid and theophylline during 180 days as compared to non-encapsulated dried green coffee extract. The highest process yield was obtained when 80% of the GA was replaced by MD and the ratio of concentrated extract to carrier agent dispersion was 1:3.5 (w/w). The optimised model obtained could be used to determine process yield, caffeic acid stability and 5-CQA retention in the microcapsules.

Keywords: chlorogenic acids, methylxanthines, caffeine, trigonelline, 5-O-caffeoylquinic acid, gum Arabic, maltodextrin

1. Introduction

Nowadays, in addition to sustenance, nourishment and enjoyment, people are increasingly looking to the non-nutrient food components for added benefits from their food, which may play a role in health promotion, disease prevention and performance improvement (Rein, Renouf, Cruz-Hernandes *et al*., 2013). Phenolic acids such as chlorogenic acids, as well as the methylxanthines caffeine and theophylline and the alkaloid trigonelline have shown numerous beneficial biological properties such as antibacterial, antioxidant, thermogenic, anticancer and hepatoprotective effects, as well as enhancing cognitive and physical performance (Vinson, Burnham, Nagendran, 2012; Shimoda, Seki, Aitani, 2006; Kono, Kobayashi, Tagawa *et al*., 1997, Koriem, Soliman, 2014; Wu, Kuo, Chen et al., 2016, Cappelletti, Piacentino, Sani *et al.*, 2015; Liao, Lee, You *et al.*, 2015), and modulating the lipid and glucose metabolism *in vivo,* both in healthy individuals and in genetically disordered metabolic conditions which affect glucose and lipid metabolism (Zhang, Chang, Liu *et al.*, 2011; Mubarak, Hodgson, Considine *et al.*, 2013).

Green coffee fruit is the major source of the phenolic acid caffeic acid and its derivatives, chlorogenic acid (CGA) and 5-caffeoylquinic acid (5-CQA), a caffeic acid ester of quinic acid which is the most abundant polyphenol in the green coffee fruit. Other important chemical compounds are caffeine and trigonelline, which are part of the major coffee components and are present in similar amounts (Perrone, Donangelo, Farah, 2008; Carvalho, Brigagão, dos Santos *et al*., 2011; Rodrigues, Salva, Bragagnolo, 2015). Thus green coffee bean is one of the best documented foods, with results from clinical studies (Vinson, Burnham, Nagendran, 2012; Soga, Ota, Shimotoyodome, 2013).

However, the effectiveness of compounds is very dependent on their preservation, bioaccessibility and bioavailability, this being the main barrier to the application of bioactive compounds in the development of nutraceuticals, since they are susceptible to degradation during processing under different conditions of temperature, light and oxygen (Khasaei, Jafari, Ghorbani *et al.,* 2014). Furthermore, the phenolic compounds need to withstand the metabolic process (pH, enzymes, microbiot, and other non-nutrient or nutrient items in the food) and be bioavailable, before they can have any effect (Favaro-Trindade, Pinho, Rocha, 2008). Another limiting factor to the application of extracts with high levels of phenolic acids is the fact that polyphenols can interact strongly with proteinaceous components (Jakobek, 2015), and furthermore, caffeine and chlorogenic acids chelate divalent ions such as Fe^{2+} and Ca^{2+} when present in the food matrix, acting as antinutritional factors (Budryn, Zaczynska, Oracz, 2016)

In order to improve the bioavailability, security and stability of functional ingredients such as vitamins, minerals, food additives, bioactive food compounds and others, both the pharmaceutical and food industries have been looking into various microencapsulation techniques which consist of the production of very small particles of liquid, solid or gaseous ingredients surrounded by different wall materials, which release their contents at controlled rates under the influence of certain stimuli (Sobrinho, Farias, 2012).

Thus the aim of this study was to develop a microencapsulated system containing whole green coffee fruit extract (GCFE) as the core, standardized for its chlorogenic acid content expressed as 5-CQA, to apply in food production as a food additive and as a bioactive substance in nutraceutical products.

2. Experimental

2.1 Materials

Chromatographic grade methanol was supplied by J.T. Backer (Phillipsburg, NJ, USA), analytical grade 99.5% ethanol was supplied by Synth (Diadema, SP, Brazil) and citric acid was supplied by Quemis (Joinvile, SC, Brazil). Ultrapure water was obtained using a Millipore system (Billerica, MA, USA), and maltodextrin (MA) (DE 4 - 7) and gum arabic (GA) were supplied by Sigma-Aldrich (St. Louis, MO, USA). The compounds 5-*O*-caffeoylquinic acid (95% pure), caffeine (99% pure), trigonelline hydrochloride (98.5% pure), caffeic acid (98% pure) and theophylline (99% pure) were supplied by Sigma-Aldrich (St. Louis, MO, USA) and used as the external standards.

2.2 Extraction process

Organically produced, fresh whole green *C. canephora* fruits of the clone code 83, were acquired from the Experimental Farm at Leopoldina (Minas Gerais, Brazil) belonging to the Company EPAMIG. The fruits were selected, washed, dried and then vacuum packed (Jumbo Plus, Selovac, SP, Brazil) until used for extraction.

The extraction was carried out under the optimized extraction conditions reported by Faria et al. (2017). Briefly, a 900 g batch of dried whole coffee fruit powder (particle size between 250 - 710 μm) was uniformly moistened with three times its weight (2.7 kg) of solvent consisting of 68% ethanol in water (w/w) for a period of 12 hours in a separate vessel (pre-

swelling phase). This material was carefully transferred and packed evenly into a 10 L stainless steel percolator (Revitec Ltda, São Paulo-SP, Brazil) and more solvent added to achieve a solid to solvent ratio of 0.9:10 (w/w), and allowed to remain in contact with the powdered material for a further 24 h (intermediate maceration phase). The flow rate of the percolator was then adjusted to 0.2±0.05 $mL.min^{-1}$ at room temperature (percolation phase). The residual solvent was removed at 40±2ºC under vacuum in a rotary evaporator Buchi® (R-220 SE model), taking care to maintain 15 % of solids in the extract before spray drying.

2.3 Preparation of microencapsulated and non-encapsulated green coffee fruit extract

A central composite rotational design (CCRD) was used to optimize the microencapsulation process, in which the process yield, encapsulation efficiency and microcapsule stability, as assessed by the bioactive compound content and reducing capacity, were chosen as the response values. Two independent variables were considered: replacement of gum arabic (GA) by maltodextrin (MD) at five levels, and the ratio of the concentrated extract to the carrier dispersion (w/w), as shown in table 1.

Different amounts of MD or GA or a mixture of both were used as the wall material so that the total solids content of the dispersion was equal to 30 % (w/w), prior to the addition of the green coffee extract. Each powder or mixture of powders was dispersed in distilled water (40ºC) and allowed to hydrate for 30 min. After dissolution, the dispersions were placed overnight in the refrigerator (5 ±1ºC) to complete the hydration process according to Silva *et al.* (2013). The next day, the concentrated green coffee extract was added to the carrier agent dispersion at five different levels as follows: 1:1; 1:1.5; 1:2.5; 1:3.5; and 1:4 (green coffee extract: carrier agent solution, w/w) and homogenized in a rotorestator homogenizer (Ultra-Turrax) at 13,600 rpm for 5 min at 30°C. The GCFE homogenised with the carrier mixture was spray-dried using a laboratory scale spray dryer.

A separate portion of the fluid green coffee extract was spray dried to produce non-encapsulated GCFE as the positive control, and the dispersion containing only gum arabic and maltodextrin (50:50 w/w) without GCFE, was spray dried as the negative control.

Table 1: Optimization of the spray-drying microencapsulation process by CCRD using the independent variables percent replacement of GA by MD (X_1) and ratio of concentrated extract: carrier agent dispersion (X_2)

Variables	-1.41	-1	0	+1	+1.41
Percent replacement of GA by MD (X_1, %)	0	20	50	80	100
Ratio of concentrated extract: carrier agent dispersion (X_2, w/w)	1:1	1:1.5	1:2.5	1:3.5	1:4

GA = gum Arabic; MD = maltodextrin;

The spray dryer (LABMAQ model MSD 1.0, Ribeirão Preto-SP, Brazil) was used with the conditions fixed as follows: feed flow rate 0.5 $L.h^{-1}$, drying air flow rate 45 $L.min^{-1}$, inlet and outlet temperatures of 160 ± 2°C and 125 ± 2ºC, respectively.

2.4 Surface morphology and particle size

The samples were mounted on the sample holder using carbon tape and then coated with gold using a Denton II Vaccum sputterer and examined usinga JEOL® model JSM – 6610 scanning electron microscope (SEM), equipped with an Energy Dispersive Spectroscope (EDS) (Thermo Scientífic NSS Spectral Imaging). SEM was carried out at 5kV with magnifications of x700, x1000 and x2500.

The particle size was measured by laser diffraction using a Malvern Mastersizer X (Ver. 2. 15) fitted with a 45mm lens. The particles were dispersed in 99.9% ethanol.

2.5 Determination of bioactive compounds by HPLC-DAD

The analysis of the bioactive compound content of the spray-dried microcapsules (MC) and the spray-dried non-encapsulated GCFE was carried out according to Robert *et al.* (2010), with some modifications in the amount of sample and the times in the ultrasonic bath and centrifuge. Briefly, twenty milligrams of microcapsules or non-encapsulated CGE were dispersed in 2 mL methanol: water: acetic acid (50:42:8 v/v/v) and agitated using a vortex mixer (1 min). The sample was then placed in an ultrasonic bath for 10 min, centrifuged at 15,000 x g for 15 min at 20ºC, and finally filtered through a nylon filter (45 µm). The extracts were quantified according to Rodrigues *et al.* (2015) using HPLC-DAD with a C18-ODS column and column guard. The mobile phase was a gradient prepared using a 10 Mm citric acid solution and methanol. The method was validated according to the International Conference on

Harmonization (ICH) (2005), evaluating the following parameters: linearity, repeatability, recovery, limits of detection (LOD) and limits of quantification (LOQ).

2.6. CGA Surface (SF) and encapsulation efficiency (EE)

The surface CGA was extracted using methanol: ethanol (1:1, v/v) according to Robert et al. (2010) with some modifications. Twenty miligram of the microcapsules were mixed with 2 mL of the mixture of methanol and ethanol (1:1 v/v), agitated in a vortex at room temperature for 1 minute and then filtered (0.45 µm Millipore filter). The CGA content present on the surface of the microcapsules ($CGA_{surface}$) was measured by HPLC-DAD using the chromatographic conditions described in item 2.5. The percent surface 5-CQA percentage was calculated according to equation 1.

$$SF\ (\%) = \frac{\text{surface 5-CQA content}}{\text{Total 5-CQA content}} \times 100 \qquad (1)$$

The encapsulation efficiency (EE, %) was determined as the ratio of the surface CGA to the total amount of CGA in the microcapsules (Equation 2).

$$EE(\%) = \frac{(CGA_{total} - CGA_{surface})}{CGA_{total}} \times 100 \qquad (2)$$

Where CGA_{total} is the total CGA content in the dried microcapsules and $CGA_{surface}$ is the amount of non-encapsulated CGA present on the surface of the microcapsules (Robert, et al., 2010).

2.7 Chlorogenic acid retention (CR)

The 5-CQA was used as a chemical marker to determine the retention of chlorogenic acid (CR %). The CR (%) was determined as the ratio of the total 5-CQA content in the microcapsules to the total 5-CQA content in the dispersions prepared (Equation 3) (Frascareli *et al.*, 2012).

$$CR\ (\%) = \frac{5\text{-CQA}total}{5\text{-CQA}initial} \times 100$$

(3)

2.8 Process Yield

The MC yield was determined as the ratio of the weight of MC (dry weight basis) collected at the dryer exit to the weight of dry matter in the dispersion taken for drying (Equation 4)

$$\text{Yield (\%)} = \frac{\text{dry mass of MC}}{\text{dry mass of dispersion}} \times 100 \quad (4)$$

2.9 Accelerated Stability Study

The spray-dried microcapsules were stored in headspace vials in a vacuum oven (Marconi, Piracicaba, Brazil) for 180 days at 40±2°C, and the accelerated shelf-life study is designed to increase the rate of chemical degradation of a dietary supplement by using exaggerated storage conditions as part of a study protocol (Stability Testing Guideline for Dietary Supplements, 2011). According to the guidelines for industries for the stability testing of new drug substances and products, as provided by the International Conference and Harmonization (ICH, 2003), the recommended storage conditions and tolerances for accelerated stability studies are 40ºC +/- 2ºC and 75% relative humidity (RH) +/- 5% RH, these being the conditions indicated for products with no special storage conditions specified on the label. In the present assay, only the temperature was controlled, since the microcapsules and non-encapsulated GCFE were stored in sealed packaging.

The water activity, reducing capacity and bioactive compound contents (5-CQA, caffeic acid, trigonelin and caffeine) were determined on day zero and during storage. Triplicate vials were withdrawn after 15, 30, 60 and 180 days of storage. Non-encapsulated spray-dried GCFE and the spray-dried carrier dispersion were used as the positive and negative controls, respectively.

2.9.1 Reducing capacity

The reducing capacity of the microcapsules and non-encapsulated GCFE were determined by the Folin–Ciocalteau colorimetric method (Singleton & Rossi, 1965). The reaction mixture contained the following components (final volume = 3000 μL): 250 μL of microcapsules or free GCFE dissolved in ultrapure water (33 and 73 $\mu g.mL^{-1}$), 1500 μL of

ultrapure water, 250 µL of Folin–Ciocalteau reagent, and 1000 µL sodium carbonate solution (2.3%, w/v).

The absorbance was monitored at 765 nm for 120 min at 25 °C, and quantification was by way of an analytical curve for gallic acid in the range between 2 and 12.5 $\mu g.mL^{-1}$, with the results expressed in milligrams of gallic acid equivalents per gram of extract (mg $GAE.g^{-1}$) according to Rodrigues *et al.* (2015).

2.9.2 Monitoring of the water activity

The water activity (Aw) of the samples was measured using a Testo® 650 thermo hygrometer (Testo AG, Lenzkirch, Germany) and a hermetic chamber.

2.10 Data analysis

The effects of the independent variables studied on the optimization of the microencapsulation process carried out according to the central composite rotational design (CCRD), were analysed using response surface methodology (RSM). A second-order polynomial equation (Equation 5) was fitted to each response to study the effects of the variables and to describe the process mathematically.

$$y = a_0 + \sum_{i=1}^{n} a_i x + \sum_{i=1}^{n} \sum_{j=i+1}^{n} a_{ij} x_i x_j + \sum_{i=1}^{n} a_{ii} x_i^2 \quad (5)$$

Where y is the predicted response, a_0 , a_i , a_{ii} and a_{ij} are the regression coefficients and x_i, x_j are the coded levels of the independent variables i and j. The model adequacy was evaluated using the F ratio and the coefficient of determination (R^2) represented at the 5% and 10% levels of significance accordingly. An analysis of variance (ANOVA), with the significance at 5% probability and the Tukey-Kramer method was applied to evaluate differences between the means amongst the different treatments, and the Pearson correlation was adopted to test the effect of the chemical stability and reducing capacity activity of the non-encapsulated and microencapsulated GCFE using GraphPad Prism 5.0 software.

3. Results and Discussion

3.1 Method validation

The calibration curves for trigonelline, caffeine, caffeic acid and 5-CQA were linear, showing R^2 values above 0.99 ($p<0.05$), and table 2 shows the results for repeatability, accuracy and values for LOD and LOQ. The accuracy of the analytical method was calculated from the recovery, which ranged from 98% to 100% for trigonelline, 99% to 102% for caffeine, 97% to 100% for caffeic acid and 98% to 102% for 5-CQA, in accordance with the values obtained by Rodrigues *et al*. (2015). The repeatability, measured as the standard deviation, was below 5%, indicating that the method was precise and accurate for the quantification of compounds from microencapsulated GCFE.

Table 2: Limit of detection (LOD), limit of quantification (LOQ), repeatability and recovery at three different spike levels for caffeine, 5-CQA, trigonelline and caffeic acid in microencapsulated whole green coffee fruit extract

Compound	LOD ($\mu g.mL^{-1}$)	LOQ ($\mu g.mL^{-1}$)	Spike level ($\mu g.mL^{-1}$)	Repeatability (%)[a]	Recovery (%)[b]
5-CQA	0.98	2.95	78.99	4.42	101.16 (3.91)
			153.21	2.82	99.96 (0.34)
			298.74	3.78	98.08 (3.25)
Caffeine	12.12	36.40	76.56	4.22	100.85 (1.08)
			147.30	1.54	101.98 (1.81)
			293.33	2.78	98.80 (1.08)
Trigonelline	3.20	9.62	28.47	4.83	100.01 (1.75)
			52.34	4.01	98.85 (1.73)
			110.22	4.19	98.34 (2.34)
Caffeic acid	2.28	6.87	0.81	2.29	99.68 (4.05)
			1.51	1.87	96.60 (3.09)
			3.11	2.36	99.82 (0.83)

5-CQA = 5-caffeoylquinic acid; [a]Repeatability is given as the relative standard deviation (RSD, %); [b]Relative standard deviation (%) is given in parentheses (n = 3).

3.2 Optimization and fitting of the model

RSM was applied to determine the effects of replacing gum arabic (GA) by maltodextrin (MD) and of the ratio of the concentrated extract to the carrier dispersion (w/w) at five levels, on the process yield, encapsulation efficiency, retention of bioactive 5-CQA, stability of the microcapsules as monitored by the water activity, reducing capacity, and the 5-CQA, caffeic acid, caffeine and trigonelline contents during 180 days of storage. Table 3 shows the results obtained for the variables of the CCRD together with the responses. Tables S1 and S2 of the

supplementary material present the regression coefficients for the fitted second order polynomial models obtained for the different responses and the ANOVA results for the model responses, respectively. In this study, the R^2 values for the three responses i.e. process yield, 5-CQA retention and caffeic acid loss after 180 days of storage were higher than 0.80, indicating a good fit and adequacy of the regression model (Joglekar & May, 1987). In addition the lack of fit was found to be non-significant ($p > 0.05$) for all the fitted models, which indicates the suitability of the model to accurately predict the variation. In this way, the selected model can be used for the simulation and optimization of variables for the encapsulation process of GCFE.

3.3 The effect of the in-process parameters

In this study a large range, from 0 to 100%, of replacement of the MD by GA in the dispersion was explored. MD is commonly mixed with GA, since MD acts protecting the core against degradation in the spray drying process due to its high glass transition temperature of around 180ºC (Drusch *et al*., 2015) and may also act as a hydrophilic sealant that limits diffusion of the core through the wall material. However, MD has no emulsifying power (Anandharamakrishnan & Padma, 2015). On the other hand, GA presents good emulsifying properties due to its molecular structure, being useful in forming rigid coatings. In addition, it helps prevent cracking of the wall when the particles are subjected to stress during atomization and drying (Cunningham, 2011; Anandharamakrishnan & Padma, 2015). For these reasons, the combination of MD together with GA has been widely explored as a coating for different core materials.

The other independent variable studied was the ratio of the concentrated extract to the carrier dispersion, exploring ratios from 1:1 to 1:4. Ouyang *et al.* (2015) found that when the core:wall ratio was greater than 6:1, the particle size was larger and the limited amount of wall material led to a thin wall which ruptured easily. The ratio of orange peel oil to gum of 1:4 provided good protection of the core against oxidation when the microcapsules were stored under appropriate water activity conditions (Beristain et al., 2002). The ratio of core to wall material of 1:4 was also determined as optimal for the production of lycopene microcapsules by spray-drying (Shu, 2006).

In this study the replacement of 80% of the GA by MD was found to be optimal for the process yield, with a minor loss of caffeic acid throughout the 180 days of storage and greater 5-CQA retention during the drying of microcapsules. In addition a ratio of concentrated extract

to carrier agent dispersion equal to 1:3.5 was found to be optimal for process yield and caffeic acid stability during storage.

In the response plot for the effect of the variables on process yield (Fig. 1A), which ranged from 49 to 61%, it was seen that a higher content of GA in the dispersion had a detrimental effect on the yield. However, the different ratios of concentrated extract to carrier dispersion (w/w) showed no significant linear or quadratic effect on the process yield. A positive interaction effect between the ratio of concentrated extract to carrier dispersion and the percentage of MD in the coating material was observed (p=0.0001), as seen in the regression equation below:

$$\text{Yield (\%)} = + 57.61 + 2.79\ x_1 - 1.72\ x_1^2 - 3.52\ x_1.x_2$$

From the response plot (Fig. 1B), it was observed that smaller concentrated GCFE to carrier dispersion ratios decreased the loss of caffeic acid, an effect that could be attributed to the optimal coating thickness provided by the carrier dispersion. The regression showed only a positive linear effect (p = 0.0359) for this independent variable, while for the percent replacement of GA by MD negative linear (0.0129) and quadratic (0.0147) effects were observed, showing no interference on the MD content on the stability of the caffeic acid. The regression equation obtained for the loss of caffeic acid during storage is given below:

$$\text{Caffeic acid content loss (mg/100g)} = 8.07 - 2.14\ x_1 + 1.67\ x_2 - 2.37\ x_2^2$$

A significant linear effect (p = 0.0000) for the replacement of GA by MD (%) was observed for 5-CQA retention (%) (Fig. 1C), showing that the increase of MD in the carrier dispersion was negative to retain 5-CQA during the spray-drying process. The regression equation for 5-CQA retention (%) is given below:

$$\text{5-CQA retention (\%)} = 68.92 - 9.99x_1$$

The models obtained for the increase in Aw during storage, for the losses in trigonelline, caffeine, 5-CQA and reducing capacity and for encapsulation efficiency (EE) were not adequate, showing values for $R^2 < 0.80$. However, for all the conditions studied, the EE was close to 100%. The microencapsulation of polyphenols from *Ilex paraguariensis* with other

coating materials such as calcium alginate and calcium alginate-chitosan gave 49–87% efficiency (Deladino, Anbinder, Navarro, & Martino, 2008), and the type of encapsulating agent and core to coating ratio have been shown to be the most important variables for the encapsulation of polyphenols (Robert et al., 2010)

According to Silva et al. (2014) the correct choice of wall material has a great influence on EE and the stability of the microcapsules. The ideal characteristics of a wall material are that it does not react with the core material, has the ability to seal and maintain the core inside the capsules, provides maximum protection to the core against adverse conditions, and, when applied to food materials, presents a neutral or non-unpleasant taste.

Carbohydrates such as GA and MD are good choices as coating materials to entrap GCFE since their interaction enhances the bioavailability of the polyphenols (Jakobek, 2015). This effect may have been mediated by the effects of carbohydrates on gastrointestinal physiology (e. g. motility and/or secretion) or by mediating the activation of a yet unidentified polyphenol-carbohydrate transporter (Schramm *et al*., 2003).

It is known that polyphenols can interact with proteins, decreasing the nutritional value of the food. However, the association of polyphenols with carbohydrates can prevent some of the negative effects of these complexes, such as the inhibition of enzyme activity or by masking the perceived astringency of some food products. Arabic gum, pectin and some other carbohydrates have shown their influence in preventing the association between grape seed polyphenols and salivary enzymes and trypsin (Gonçalves, Mateus & de Freitas, 2011; Soares, Mateus & de Freitas, 2012).

A number of studies have indicated various beneficial effects of the association of polyphenols with carbohydrates in the large intestine (MacDonald & Wagner, 2012; Palafox-Carlos *et al*., 2011; Saura-Calixto, 2011; Tuohy *et al*., 2012). When protected by carbohydrates from the gastrointestinal pH and action of enzymes, the polyphenols can reach the large intestine where they show several different positive effects such as enhancement of their bioaccessibility in the colon, which positively affect the growth of colon microbiota. In addition, microorganisms present in the digestive tract can metabolize the polyphenolic compounds releasing metabolites which can exhibit various positive effects (MacDonald & Wagner, 2012; Palafox-Carlos *et al*., 2011; Saura-Calixto, 2011; Tuohy *et al*., 2012). In general polyphenols and theis metabolits can create a positive antioxidant environment in the colon (Saura-Calixto, 2011). Activities commonly described for the metabolites of polyphenols are antibacterial and anti-inflammatory activities and detoxification processes (Tuohy *et al*., 2012),

and the most positive role relates to protection against the risk of developing colorectal cancer (MacDonald & Wagner, 2012).

The suitability of the model for the responses was determined under the optimum conditions for the replacement of GA by MD (80%) and for the ratio of concentrated extract to carrier dispersion (1:3.5, w/w). The experimental values for the responses were found to compare well and be in agreement with the predicted values (Table S3).

3.4 Accelerated stability study

To monitor the stability of the powders as recommended by the Stability Testing Guideline for Dietary Supplements (2011), the water activity, alterations in the bioactive compound contents and changes in the *in vitro* reducing capacity activity of the microcapsules and non-encapsulated GCFE were analysed at five points in time.

3.4.1 Effect of storage on the water activity

The water activity is an essential parameter for powder safety and stability since it determines the shelf life of the product (Tonon *et al*., 2008). Water adsorption is an important process during the storage of powders and can lead to changes in their chemical properties that can negatively affect the quality of many food products (Pitalua et al., 2010). When the Aw is below 0.3, there is a reduction in the reaction rate, and the food product can be considered stable (Fennema, 1996), except in terms of lipid oxidation which was not considered in this study, since the core contained no lipid compounds.

Amongst the treatments evaluated in the present study, including the non-encapsulated GCFE and the negative control, the water activity ranged from 0.07 to 0.14. The treatments differed significantly from each other at zero time (T0), with the exception of the central points, microparticles obtained from treatment 7, and the negative control particles, which did not differ significantly from each other (data not shown). The latter samples were spray-dried with 50% of MD. The Aw of encapsulated anthocyanins obtained from *Ipomoea batata* was significantly affected by the different ratios of MD to GA. Nawi *et al*. (2015) showed the lowest Aw values for combinations of GA and MD as the wall material, followed by those produced with MD alone. On the other hand, particles produced with GA alone showed the highest water activity values. However, this behavior was not observed amongst the treatments in the present

study, where the microparticles produced with GA alone initially showed the lowest Aw value as compared to those produced with 100% MD ($P<0.001$), and for the treatments with combined GA and MD, the higher the content of MD the higher the Aw value at T0 ($p<0.05$), as can be seen in supplementary figure 1.

After 180 days of storage the highest Aw values were about 0.3, values found for the non-encapsulated GCFE followed by the MCs from treatment 3. All the other treatments were different from the non-encapsulated GCFE and MC3 treatment, showing lower Aw values. The increase in water activity appears to have been influenced by the lower MD content in the carrier dispersion and the ratio of concentrated GCFE to carrier dispersion, due to the formation of a thinner wall protecting the core. However, these effects were not observed in the response surface method.

3.4.2 Effect of storage on the reducing capacity activity and on the chemical composition

Table S4 of the supplementary material shows the bioactive compound contents of the microcapsules and non-encapsulated GCFE as measured by HPLC-DAD. The products were analyzed at five different storage times in a vacuum oven at 40 ± 2ºC, first at zero time (T0), and then after 15 (T1), 30 (T2), 60 (T3) and finally 180 (T4) days.

Caffeic acid is highly hygroscopic and degrades at room temperature and in the presence of light (Khan *et al*., 2016). In overall, the content of this compound decreased significantly in the particles during storage ($p>0.05$), as observed by RSM, except for MC2, where the caffeic acid content was stable for 180 days. As can be seen from the RSM results, the microparticles formulated with a greater MD percentage in the carrier dispersion and a smaller concentrated extract to carrier dispersion ratio, showed greater stability in relation to the caffeic acid content.

The 5-CQA content was significantly stable ($p<0.05$) in the MC2, MC5 and MC6 treatments but showed a significant difference one from the other in the other treatments. The non-encapsulated GCFE only showed a significant change in the 5-CQA content in the last HPLC analysis after 180 days of storage. The thermal decomposition behaviour of 5-CQA under inert (nitrogen) and oxidative (air) conditions demonstrated that 5-CQA suffered its first decomposition at 201 ± 1ºC under both atmospheric conditions, using a heating rate of 1 $^{\circ}C.min^{-1}$ (Owusu-Ware et al., 2013). These results show that 5-CQA is a very heat stable compound, even under different atmospheric conditions.

The non-encapsulated GCFE showed the same behaviour as the other treatments for the compounds caffeine, trigonelline and caffeic acid, but theophylline showed a significant

decrease as from 15 days after storage (T1). Amongst the different treatments, caffeine and trigonelline showed slight decreases, with significant differences only after 15 or 30 days of storage ($p>0.05$). The contents of these compounds were stable for 180 days in MC2, MC6 and MC8 ($p<0.05$). In agreement with Wesolowski & Szynkaruk (2008), the methylxanthines showed melting points as from 235°C in the case of caffeine to 272 ºC in the case of theophylline, with heating rates of 10°C min^{-1}. Thus free methylxanthines melt and decompose at high temperatures (Wesolowski & Szynkaruk, 2008).

The stability of the chlorogenic acids and caffeine from the green coffee extract was tested in different model food systems, heating for 0.5 and 1h at 110ºC for model systems containing sucrose and potato starch, and at 180ºC for model systems with egg white protein and sunflower oil. The chlorogenic acid losses were shown to range from 18 to 84 % and those of caffeine from 1.5 to 10 %. The greatest green coffee polyphenol degradation took place during heating of the GCFE with sunflower oil and the smallest losses were observed in the sucrose system, followed by the protein system. However, relatively high losses of chlorogenic acids are expected in a protein system, due to polyphenol-protein interactions. With respect to caffeine losses, no clear trend was established between the caffeine losses and the type of system or heating time (Budryn *et al*., 2013).

The above results found by Budryn et al. (2013) show the importance of protecting the green coffee extract using the microencapsulation process, in order to apply this product to a food matrix that may or may not be processed, since even without the heating process the polyphenols and caffeine can interact with the proteins and bivalent metals present in the food system (Jakobek 2015; Budryn, Zaczynska, Oracz, 2016).

Table 3: CCRD for the microencapsulation parameters (independent variables, X_1 and X_2) and the responses for encapsulation efficiency (EE, %), process yield, 5-CQA retention (%), increase in Aw, loss in reducing capacity, and losses in the 5-CQA, trigonelline and caffeine contents after 180 days of storage (dependent variables)

	Variables		Responses								
Run	(X_1, %)	(X_2, w/w)	Increase in Aw after 180 days	EE (%)	Yield (%)	Loss of reducing capacity after 180 days (mg GAE.g^{-1} of sample)	Loss of 5-CQA content after 180 days (mg/100g)	Loss of caffeine content after180 days (mg/100g)	Loss of trigonelline content after180 days (mg/100g)	Loss of caffeic acid content after 180 days (mg/100g)	5-CQA retention (%)
1	20 (-1)	1:1.5 (-1)	0.191±0.009	99.14±0.04	48.71	23.6±0.7	264±12	437±17	77±3	3.5±0.1	71±1
2	80 (+1)	1:1.5 (-1)	0.128±0.007	99.30±0.094	61.08	18.6±0.4	331±13	155±5	151±9	1.4±0.1	60±2
3	20 (-1)	1:3.5 (+1)	0.125±0.007	98.31±0.024	56.93	18.4±0.9	229 ±10	142±1	89±3	8.4±0.2	78±3
4	80 (+1)	1:3.5 (+1)	0.141±0.007	99.73±0.02	55.21	22.4±1.0	254±8	164±4	148±3	6.1±0.7	59±3
5	0 (-1.41)	1:2.5 (0)	0.160±.0.010	98.80±0.04	50.45	10.2±0.4	144±7	102±2	159±6	11.5±0.9	87±2
6	100 (+1.41)	1:2.5 (0)	0.156±0.010	99.49±0.07	58.72	16.9±0.6	50±1	114±2	80±3	2.5±0.2	52±1.5
7	50 (0)	1:4 (+1.41)	0.129±0.007	99.60±0.02	57.86	9.9±0.6	145±3	92±1	68±3	2.8±0.2	70±1
8	50 (0)	1:1 (-1.41)	0.148±0.006	98.04±0.08	56.75	12.9±0.5	319±17	161±5	107±6	5.5±0.1	72±1
9	50 (0)	1:2.5 (0)	0.150±0.019	99.03±0.02	58.94	23.5±1.5	141±7	74±2	56±2	9.3±0.3	69±1
10	50 (0)	1:2.5 (0)	0.160±0.004	99.01±0.08	57.67	23.3±0.7	127±5	74±5	41±2	9.3±0.3	70±2
11	50 (0)	1:2.5 (0)	0.149±0.005	98.88±0.03	57.67	23.6±0.7	164±6	89±4	49±2	9.2±0.6	68±0.5

Aw = Water activity; 5-CQA = 5-*O*-caffeoylquinic acid; X_1 = Percent replacement of gum Arabica by maltodextrin (%); X_2 = Ratio concentrated extract: carrier dispersion (w/w)

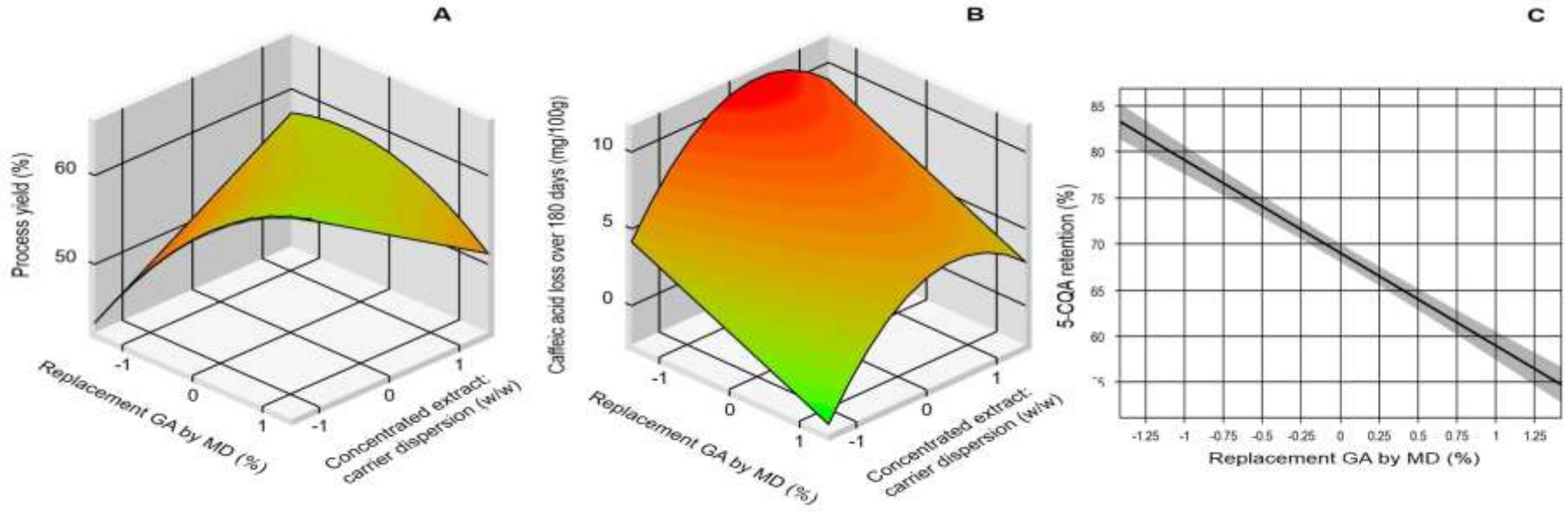

Fig 1: Response surface optimization for two responses (A) process yield, (B) caffeic acid loss during storage, and linear response for (C) 5-CQA retention.

A previous study that assessed the influence of the inclusion of chlorogenic acids with β-cyclodextrin on the antioxidant capacity and bioavailability indicated that its inclusion did not limit these parameters (Paramera, Konteles, & Karathanos, 2011). On the other hand, Budryn et al. (2014) showed that crude and purified extracts obtained from green Robusta coffee beans showed an antioxidant capacity that was about 2-fold higher than that of the complex obtained, but the product stability was not assessed by the authors.

The effect of temperature and time on the reducing capacity was analysed by ANOVA with a 95% confidence interval and showed a non-significant ($p<0.05$) loss of reducing capacity amongst the different treatments during storage, including that of the non-encapsulated GCFE, with the exception of MC8, which showed a significant difference as compared to MC4 ($P>0.05$). However, considering the same treatment, the reducing capacity was affected by storage, as can be seen in table 4 ($p>0.05$).

The decrease in reducing capacity amongst the treatments ranged from 9.80 to 23.60 mg $GAE.g^{-1}$ of sample. The reducing capacity of the non-encapsulated GCFE decreased about 30% after 180 days of storage as did the reducing capacities of MC3 and MC4, which showed decreases of 31 and 30% respectively, whilst MC8 and MC7 showed decreases of only 11 and 14%, respectively (supplementary figure 2). However, using response surface methodology, no effect on this parameter was found for the independent variables tested ($p<0.05$).

The encapsulation of essential cloves oil with β-cyclodextrin provided greater stability and increased bioaccessibility of the total phenolic compounds and antioxidant capacity (Cetin Babaoglu et al., 2017). However, in comparison with other encapsulating systems, the loading capacity of the inclusion complexes provided by β-cyclodextrin is low (generally 1:1 M ratio with the payload molecule) and the cost of the material is high (Atefi *et al*., 2017).

The relationship between the antioxidant capacity and specific phenolic compounds was unclear. A positive Pearson's correlation of >0.80 ($p\leq0.05$) was confirmed for the losses of 5-CQA, theophylline and caffeine contents and of reducing capacity during storage, but this was not confirmed for the losses of caffeic acid and trigonelline contents <0.80 ($p\geq0.05$). This lack of correlation between the loss of some phenolic compounds and antioxidant capacity was also reported by other authors in different food systems (Kotseridis *et al*., 2013; Rocha-Parra *et al.*, 2016). When discussing the reducing or antioxidant capacity, one must also mention the contribution of caffeine and other methylxanthines to the antioxidant activity in complex systems (Devasagayam *et al*, 1996; Farah, 2012), which could be positively observed during this stability study.

When considering the results of the present study, it appears that the microencapsulation process did not improve the stability of the green coffee extract, since no significant difference could be seen between the microcapsules and the non-encapsulated GCFE in relation to the stability of the bioactive compounds.

3.5 Surface morphology and particle size

The different formulations had no effect on the general appearance of the microcapsules. Furthermore, figure 2 showed that the particle size became smaller when the concentrated extract to carrier dispersion ratio increased from 1:4 to 1:1, considering the same proportion of MD and GA in the carrier dispersion. The samples with a core to coating ratio of 1:1 had a smaller amount of coating material, which led to better mixing and a higher energy density as compared to core to coating ratios of 1:4. As a result, smaller particles were obtained for samples with a core to coating ratio of 1:1.

The bi-dimensional surface characteristics of the powder particles as observed by SEM showed that 100% maltodextrin (3F, MC6) was the encapsulating agent that enabled the formation of more homogeneous microcapsules (Figure 2). The non-encapsulated GCFE powder particles showed smooth surfaces and a homogeneous size.

The microparticles obtained with 100% of MD showed similar structures to those obtained with different amounts of MD/GA, with few wrinkles and a smooth surface, as also found by Silva *et al.* (2013) when they used MD:GA as the encapsulating agent for a jaboticaba peel extract. However, the particles obtained with 100% of GA exhibited an irregular surface and angular shapes with several indentations. The indentations on the surfaces of spray dried particles are usually attributed to particle shrinkage due to the drastic loss of moisture followed by cooling (Saénz *et al*., 2009).

The particles size distribution was measured using a laser light diffraction system (Mastersizer S, Malvern Instruments, Malvern, UK), and the particle size (*D[4,3]*) was expressed as the mean of six repetitions. According to Gong *et al*. (2008), spray-dried powders often have a small particle size (<50 μm), and in the present study, 90% of all MC obtained were smaller than 27 μm and 10 % were greater than 1 μm (Table 4).

Of the non-encapsulated GCFE, 90% of the particles were smaller than 4 μm. In agreement with that reported by Santana *et al*. (2013), a particulate system with small particles is less porous, and thus liquid penetration into such a system is more difficult, resulting in poor reconstitution properties. The addition of maltodextrin can improve powder reconstitution,

since it is highly water soluble and of low viscosity (even at high solid concentrations) (Bakowska-Barczak & Kolodziejczyk, 2011), features required when choosing a spray drier aid to obtain good quality, stable particles.

Table 4: Particle size analysis of spray-dried microencapsulated GCFE.

Microcapsules	d_{90}[a] (µm)	d_{10} (µm)	d_m (µm)	Range (µm)	Span (-)	Vol. (weighted mean) D[4,3]:
MC1	26.6±0.4	2.18±0.03	11.2±0.1	24.4±0.4	2.16±0.04	13.2±0.2
MC2	15.2±0.3	1.37±0.01	6.8±0.1	13.8±0.2	2.03±0.00	7.7±0.1
MC3	22.7±0.2	1.78±0.30	9.7±0.1	20.9±0.3	2.13±0.01	11.2±0.1
MC4	21.5±0.2	1.92±0.02	9.3±0.1	19.6±0.2	2.11±0.00	11.1±0.1
MC5	25.3±0.2	2.05±0.03	10.4±0.1	23.3±0.2	2.24±0.01	12.3±0.1
MC6	15.5±0.1	1.57±0.00	7.5±0.0	13.9±0.1	1.86±0.00	8.2±0.03
MC7	19.8±0.2	2.01±0.01	9.2±0.1	17.7±0.2	1.93±0.00	10.3±0.1
MC8	12.6±0.1	1.19±0.00	5.3±0.0	11.4±0.1	2.14±0.01	6.3±0.7
CP	14.68±0.18	1.48±0.04	6.6±0.1	13.2±0.2	2.01±0.02	7.5±0.1

[a]d_{90} cumulative frequency at 90%; d_{10} cumulative frequency at 10%; d_m mean diameter. Results are expressed as the mean ± standard deviation (SD). MC = microcapsules, CP = central points (MC9, MC10 and MC11).

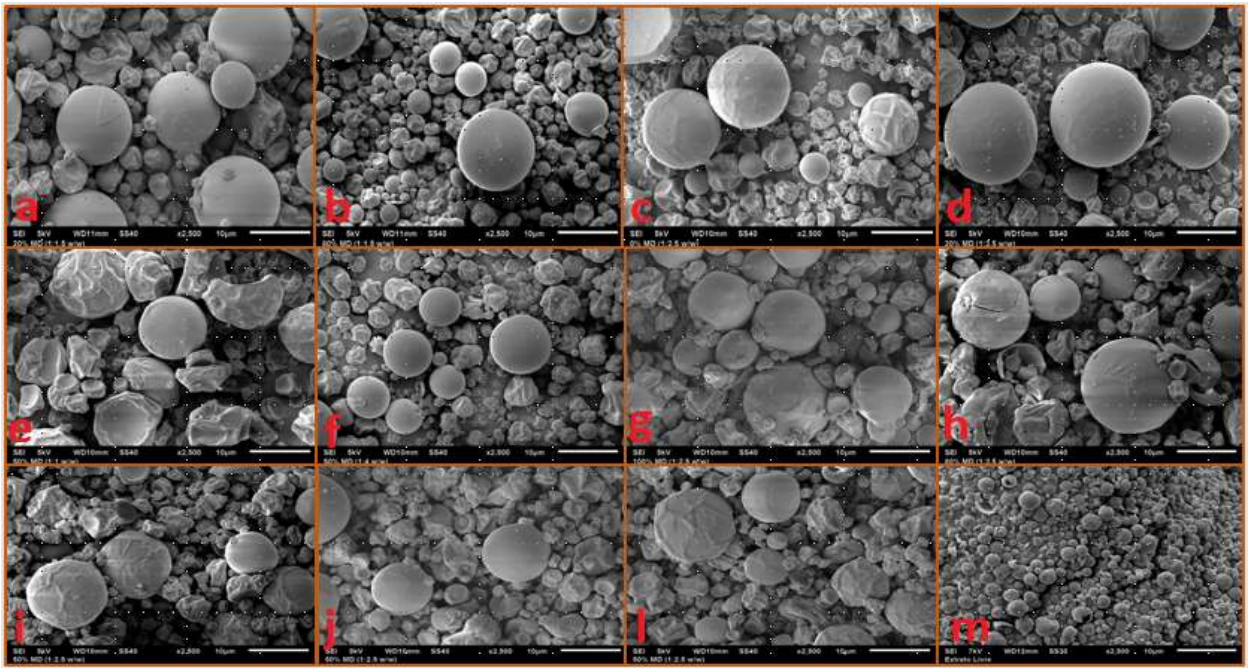

Fig 2: Surface photomicrography by scanning electron microscopy of microencapsulated and non-encapsulated GCFE (2500 x 5 KV). a = MC1, b = MC2, c = MC3, d = MC4, e = MC5, f = MC6, g = MC7, h = MC8, i = MC9, j = MC10, l = MC11, m = non-encapsulated GCFE.

4. Conclusion

By maximizing the response of process yield and minimizing the responses of overall bioactive compound content, stability, Aw and 5-CQA retention in the microencapsulation of concentrated GCFE with different percentages of MD and GA as the carrier agent and different ratios of carrier agent to core in the formulated dispersion, the use of 80% of MD and a concentrated extract to carrier dispersion ratio equal to 1:3.5 (w/w) was recommended as the condition for simultaneous optimization.

Since the process yield is an important parameter in industrial processing and the parameter of stabilitywas not significantly different between the treatments, the use of the desirability function was successfully employed to optimize the independent variables studied, in order to produce microparticles that represent a promising additive for application in the pharmaceutical, dietary and cosmetic industries due to the presence of phenolic acids and caffeine. Furthermore, since the microencapsulation process did not significantly alter the stability of the GCFE, the non-encapsulated product could be applied as a supplement in non-food products.

According to the microstructural analysis of the microspheres obtained, both the use of maltodextrin alone and the mix of gum Arabic with maltodextrin (50 - 80%) allowed for the formation of more homogeneous particles, which is recommended for the spray-drying microencapsulation process.

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Supplementary material

Tables S1

Regression coefficients of the fitted second order polynomial for the three responses and their significance

Coefficient	Yield	Caffeic acid loss after 180 days	5-CQA retention
X_0	58.09[a]	9.31[a]	69.33[a]
X_1	2.79[a]	-2.14[c]	-9.99[a]
X_1^2	-1.87[a]	-1.32	-0.65
X_2	0.49	1.67[c]	0.38
X_2^2	-0.51	-2.75[c]	0.08
$X_1.X_2$	-3.52[a]	-0.04	-1.98

** [a] significant at 0.1% ($p<0.001$); [b]significant at 1% ($p<0.01$); [c] significant at 5% ($p<0.05$)

Table S2

ANOVA values for the fitted models and lack of fit

	df	Sum of squares		
		Yield	Caffeic acid loss after 180 days	5-CQA retention
Model	5	133.7	103.5	818.6
Lack of fit	3	0.6	13.6	85.3
Pure error	2	1.1	0.0	1.8
R^2	---	0.99	0.88	0.90

Table S 3

Optimized microcapsules obtained using the response optimizer

Optimal variables		**Predicted value**			**Actual value**		
Replacement MD by GA (X_1, %)	**Rate concentrated extract: carrier agent dispersion (X_2, w/w)**	**Yield (%)**	**Caffeic acid loss after 180 days (mg.100g^{-1})**	**5-CQA retention (%)**	**Yield (%)**	**Caffeic acid loss after 180 days (mg.100g^{-1})**	**5-CQA retention (%)**
80%	1:3.5	61.93	6.45	59.34	61.08	6.12±0.72	60±1.85
80%	1:1.5		1.47			1.45±0.10	
0%	1:2.5			82.34			87.32±2.27

MD = maltodextrin; GA = gum arabic

Figure S1

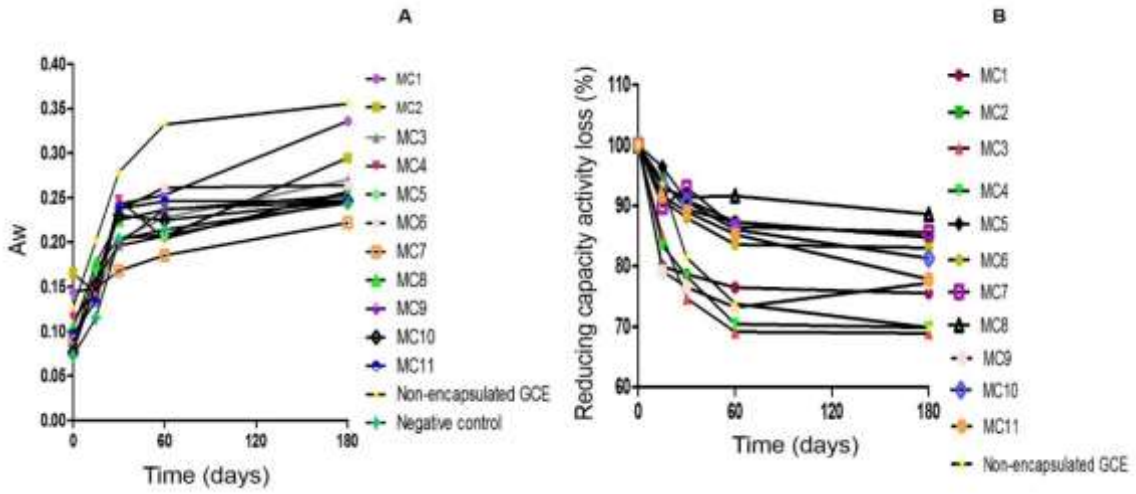

Fig 1. Increase of water activity (A) and reducing capacity activity loss (B) of the microcapsules, non-encapsulated GCFE and negative control during the 180 days of storage.

Figure S2

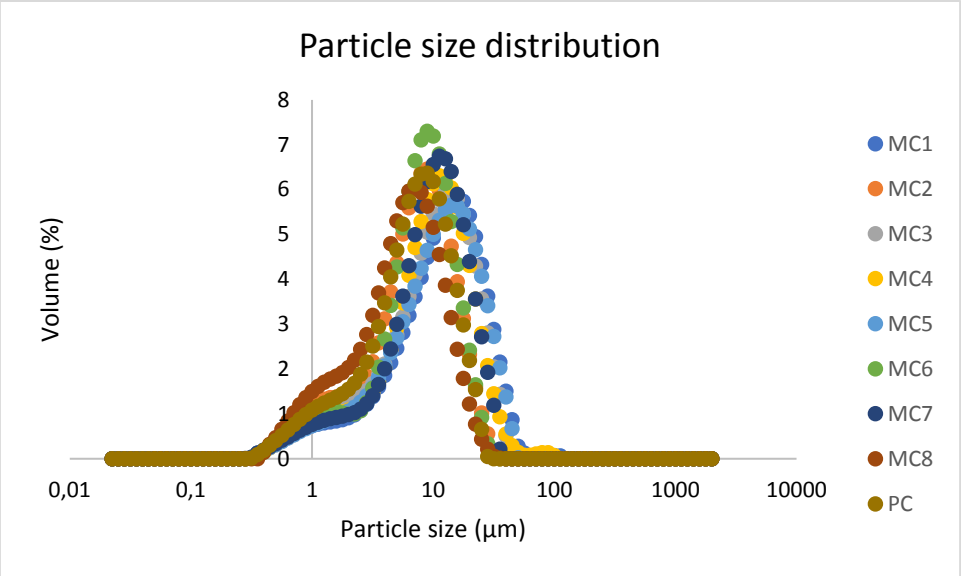

Fig 2. Particle size distribution obtained by optimization process. MC = microcapsules PC = Central points

Table S4

Real values of bioactives content and reducing capacity activity of microcapsules and non-encapsulated GCFE monitored during 180 days at four different times.

5-CQA (mg.100g^{-1} d.b.)			Stability storage (days)				
(X1, %)	(X2, *w/w*)	Run	0 day	15 days	30 days	60 days	180 days
20 (-1)	1:1.5 (-1)	MC1	3009±40^{a}	2949±5^{a}	2828±146^{ab}	2822±77^{ab}	2744±107^{b}
80 (+1)	1:1.5 (-1)	MC2	3092±97	3011±120	3003.7±102	2911.2±48	2859±41
20 (-1)	1:3.5 (+1)	MC3	1290±48^{a}	1267±54^{a}	1129±9^{ab}	1059±39^{b}	1061±133^{b}
80 (+1)	1:3.5 (+1)	MC4	1421±52^{a}	1289±17^{b}	1250±22^{bc}	1234±16^{bc}	1167±42^{c}
0 (-1.41)	1:2.5 (0)	MC5	1620±44	1581±139	1507±79	1492±33	1476±31
100 (+1.41)	1:2.5 (0)	MC6	1893±41	1867±143	1852±69	1817±61	1842±36

50 (0)	1:4 (-1.41)	MC7	1143±36^{a}	1121±12^{ab}	1091±8^{bc}	1028±75^{bc}	998±37^{c}
50 (0)	1:1 (+1.41)	MC8	4332±29^{a}	4261±73^{a}	4221±13^{ab}	4169±65^{b}	4013±43^{c}
50 (0)	1:2.5 (0)	MC9	1645±28^{a}	1596±17^{ab}	1592±30^{ab}	1554±50^{ab}	1504±38^{b}
50 (0)	1:2.5 (0)	MC10	1646±68	1688±12	1589±7	1555±102	1519±82
50 (0)	1:2.5 (0)	MC11	1655±77^{a}	1635±17^{a}	1582±49^{ab}	1501±25^{b}	1491±56^{b}
Positive control		Non-encapsulated GCFE	13174±223^{a}	13026±97^{a}	12859±74^{a}	12758±135^{a}	11828 ±346^{b}

Caffeine (mg.100g^{-1} d.b.)

20 (-1)	1:1.5 (-1)	MC1	2084±24^{a}	2047±16^{a}	1989±30^{a}	1786±89^{b}	1647±33^{c}
80 (+1)	1:1.5 (-1)	MC2	2008±68	2001±36	1958±61	1948±34	1941±146
20 (-1)	1:3.5 (+1)	MC3	1061±27	100±92	948±2.4	922±20	918±85
80 (+1)	1:3.5 (+1)	MC4	1163±34^{a}	1054±23^{b}	1048±11^{b}	1039±13^{b}	998±17^{b}
0 (-1.41)	1:2.5 (0)	MC5	1363±62^{a}	1189±53^{b}	1276±31^{ab}	1321±88^{ab}	1261±12^{ab}
100 (+1.41)	1:2.5 (0)	MC6	1376±9.7	1343±46	1344±10	1290±96	1262±47
50 (0)	1:4 (-1.41)	MC7	1021±23^{a}	998±4^{a}	989±5^{a}	966±30^{ab}	929±29^{b}
50 (0)	1:1 (+1.41)	MC8	2970±12	2924±167	2907±10	2888±45	2809±33
50 (0)	1:2.5 (0)	MC9	1356±18^{a}	1315±12^{ab}	1323±17^{ab}	1312±40^{ab}	1282±21^{b}
50 (0)	1:2.5 (0)	MC10	1481±43^{a}	1460±20^{ab}	1441±27^{ab}	1438±16^{ab}	1407±8^{b}
50 (0)	1:2.5 (0)	MC11	1505±28^{a}	1489±4^{a}	1484±23^{a}	1470±10^{a}	1416±11^{b}
Positive control		Non-encapsulated GCFE	8805±155^{a}	8650±23^{a}	8626±39^{ab}	8546±28^{ab}	8307±217^{b}

Trigonelline (mg.100g^{-1} d.b.)

20 (-1)	1:1.5 (-1)	MC1	868±11^{a}	866±11^{a}	864±38^{a}	792±25^{b}	791±21^{b}
80 (+1)	1:1.5 (-1)	MC2	851±12^{a}	838±10^{ab}	752±62^{bc}	715±43^{c}	700±4^{c}
20 (-1)	1:3.5 (+1)	MC3	436±14^{a}	444±14^{a}	440±29^{a}	358±12^{b}	347±7^{b}
80 (+1)	1:3.5 (+1)	MC4	439±14^{a}	412±16^{a}	417±20^{a}	366±4^{b}	292±11^{c}

0 (-1.41)	1:2.5 (0)	MC5	558±14^{a}	533±23^{a}	534±12^{a}	515±22^{a}	459±23^{b}
100 (+1.41)	1:2.5 (0)	MC6	538±10	535±44	533±30	504±17	473±18
50 (0)	1:4 (-1.41)	MC7	387±11^{a}	388±5^{a}	365±24^{ab}	329±16^{bc}	319±9^{c}
50 (0)	1:1 (+1.41)	MC8	1179±14	1113±111	1167±37	1096±27	1072±7
50 (0)	1:2.5 (0)	MC9	531±5^{a}	517±4^{a}	514±16^{a}	470±15^{b}	475±11^{b}
50 (0)	1:2.5 (0)	MC10	537±11	531±5	528±10	517±31	497 ±24
50 (0)	1:2.5 (0)	MC11	535±27	533±25	530±5	521±24	486±4
Positive control		Non-encapsulated GCFE	3470±42^{a}	3418±35^{ab}	3489±47^{a}	3290±33^{b}	3213±107^{b}

Caffeic acid (mg.100g^{-1} d.b.)

20 (-1)	1:1.5 (-1)	MC1	21.2±0.8^{a}	19.2±0.6^{b}	18.6±0.9^{b}	17.5±0.4^{b}	17.7 ±0.9^{b}
80 (+1)	1:1.5 (-1)	MC2	25±0.7	25.8±1.0	24.9±0.6	24.2±0.3	23.6±2.5
20 (-1)	1:3.5 (+1)	MC3	10.6±0.1^{a}	5.1±0.2^{b}	3.0±0.2^{c}	2.3±0.2^{d}	2.2±0.2^{d}
80 (+1)	1:3.5 (+1)	MC4	10.8±0.6^{a}	6.5±0.1^{b}	6.4±0.1^{b}	6.4±0.2^{b}	4.7±0.1^{c}
0 (-1.41)	1:2.5 (0)	MC5	16.1±0.7^{a}	7.9 ±0.2^{b}	6.2±0.2^{c}	4.7±0.3^{d}	4.6±0.4^{d}
100 (+1.41)	1:2.5 (0)	MC6	16.2±0.1^{a}	16.0±0.3^{a}	13.0±0.4^{b}	12.5±0.7^{b}	13.6±0.9^{b}
50 (0)	1:4 (-1.41)	MC7	3.8±0.1^{a}	3.6±0.1^{b}	3.8±0.1^{ab}	2.8±0.1^{c}	1.1±0.1^{d}
50 (0)	1:1 (+1.41)	MC8	39.9±0.8^{a}	36.3±1.7^{bc}	38.3±0.5^{ab}	38.4±1.4^{ab}	34.3±1.4^{c}
50 (0)	1:2.5 (0)	MC9	16.4±0.4^{a}	8.9±0.2^{b}	8.5±0.4^{b}	8.2±0.4^{b}	7.1±0.2^{c}
50 (0)	1:2.5 (0)	MC10	16.8±0.9^{a}	11.5±0.5^{bc}	12.0±0.1^{b}	12.6±0.1^{b}	10.1 ±0.5^{c}
50 (0)	1:2.5 (0)	MC11	16.4±0.9^{a}	9.9±0.3^{b}	10.1±0.5^{b}	8.8±0.4^{b}	7.1 ±0.5^{c}
Positive control		Non-encapsulated GCFE	155±3.4^{a}	153±0.3^{a}	153±4.0^{a}	150±0.6^{a}	136±5.8^{b}

Theophylline (mg.100g^{-1} d.b.)

20 (-1)	1:1.5 (-1)	MC1	8.9±0.4^{a}	6.3±0.3^{b}	6.7±0.1^{b}	6.70±0.18^{b}	n.d^{c}
80 (+1)	1:1.5 (-1)	MC2	9.6±0.3^{a}	8.6±0.4^{a}	9.4±0.2^{a}	9.3±0.3^{a}	7.3±0.6^{b}

20 (-1)	1:3.5 (+1)	MC3	4.9±0.3^{a}	4.6±0.3^{b}	2.2±0.0^{c}	2.5±0.1^{c}	0.9±0.1^{d}
80 (+1)	1:3.5 (+1)	MC4	4.8±0.2^{a}	2.9±0.0^{b}	2.3±0.1^{cd}	2.2±0.1^{c}	1.8±0.1^{d}
0 (-1.41)	1:2.5 (0)	MC5	5.5±0.3^{a}	4.9±0.0^{b}	3.4 ±0.1^{c}	3.7±0.1^{c}	2.9±0.3^{d}
100 (+1.41)	1:2.5 (0)	MC6	5.3±0.1^{a}	4.4±0.4^{a}	3.3±0.1^{b}	3.6±0.2^{b}	3.2±0.2^{b}
50 (0)	1:4 (-1.41)	MC7	3.0±0.0^{a}	3.2±0.3^{a}	2.1±0.1^{b}	1.7±0.1^{b}	1.7±0.1^{b}
50 (0)	1:1 (+1.41)	MC8	17.9±0.6^{a}	15.8±0.3^{b}	14.1±0.2^{c}	14.6±0.9^{bc}	11.1±0.3^{d}
50 (0)	1:2.5 (0)	MC9	5.4±0.1^{a}	5.0±0.2^{a}	5.3±0.2^{a}	4.2±0.3^{ab}	3.2±0.2^{b}
50 (0)	1:2.5 (0)	MC10	5.1±0.2^{a}	5.1 ±0.1^{a}	4.9±0.3^{ab}	4.9±0.1^{a}	4.2 ±0.4^{b}
50 (0)	1:2.5 (0)	MC11	5.01±0.1^{a}	4.8±0.2^{a}	5.0±0.1^{a}	4.5±0.1^{a}	3.67±0.3^{b}
Positive control		Non-encapsulated GCFE	63.9±1.2^{a}	57.7±0.1^{b}	53.1±1.1^{c}	43.9±1.7^{d}	34.7±1.5^{e}
Reducing capacity (mg GAE.g^{-1} sample)							
20 (-1)	1:1.5 (-1)	MC1	113±3.0^{a}	90 ±3.1^{b}	89±3.8^{b}	90±4.4^{b}	89 ±4.0^{b}
80 (+1)	1:1.5 (-1)	MC2	102±0.2^{a}	96±6.0^{ab}	91±1.4^{bc}	89± 3.3^{bc}	86.34 ±3.9^{c}
20 (-1)	1:3.5 (+1)	MC3	58±1.2^{a}	49±2.5^{b}	43 ±4.2^{bc}	40±2.6^{c}	40±1.0^{c}
80 (+1)	1:3.5 (+1)	MC4	68±1.5^{a}	57 ±1.1^{b}	53 ±4.1^{bc}	48. ±2.1^{c}	48 ±2.8^{c}
0 (-1.41)	1:2.5 (0)	MC5	71.3±0.7^{a}	68.1±2.2^{ab}	64.3±5.4^{ab}	62±3.0^{b}	60±2.6^{b}
100 (+1.41)	1:2.5 (0)	MC6	73±6.2^{a}	67±4.0^{ab}	64±2.6^{ab}	61.3±1.7^{b}	61±3.5^{b}
50 (0)	1:4 (-1.41)	MC7	54±0.9^{a}	48±2.7^{ab}	50±2.3^{ab}	47 ±3.1^{b}	46±1.5^{b}
50 (0)	1:1 (+1.41)	MC8	150 ±5.1^{a}	140±5.5^{ab}	138±6.2^{ab}	138±6.9^{ab}	133±1.5^{b}
50 (0)	1:2.5 (0)	MC9	87±0.6^{a}	69±2.6^{b}	67±2.8^{bc}	64±1.0^{bc}	61±4.4^{c}
50 (0)	1:2.5 (0)	MC10	82±3.3^{a}	77±2.3^{ab}	74±2.7^{abc}	71±3.2^{bc}	67±3.3^{c}
50 (0)	1:2.5 (0)	MC11	85±3.7^{a}	75±2.1^{b}	73±4.7^{b}	70±3.2^{bc}	64±1.8^{c}
Positive control		Non-encapsulated GCFE	565±40^{a}	523±17^{ab}	461±31^{bc}	417±25^{c}	395±43^{c}

Values obtained at different storage times for each bioactive compound and reducing capacity followed by different letters in the horizontal lines are significantly different ($p < 0.05$). nd = not detected. MC = microcapsules

CAPITULO V

In silico toxicity prediction and antioxidant activity of microencapsulated and non-encapsulated spray-dried extract from green coffee fruit Arabica and Robusta

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ABSTRACT

The hydroethanolic extracts from green coffee fruit Arabica and Robusta (GCFE) were obtained by percolation using 68% of ethanol and 0.9:10 (w/w) proportion of solid to solvent and then the fluid extracts were dried by spray drier in the shape of free extracts or microencapsulated using maltodextrin (MD) and gum arabic (GA) (1:1, w/w) as wall agents. The encapsulation efficiency (EE, %) was 96.9±0.04 and 97.36±0.03% and the chlorogenic acids retention 59.61±1.3 and 73.72±2.49 to arabic and Robusta GCFE, respectively. Quantification of GCFE bioactives was carried out by HPLC-DAD and compound identification was obtained by HPLC-DAD-MS^n. A higher content of total chlorogenic acids and caffeine was found in Robusta coffee extracts, but the trigonelline content was higher in Arabica GCFE. Predictions of toxicity from major phytoconstituents found in GCFE were performed via computational methods. The ACD/I-Lab, AdmetSAR, and pKCSM computational tools showed that GCFE major compounds revealed no toxicological potential for most of the parameters assessed, including Ames toxicity, carcinogenicity, hERG inhibition, hepatotoxicity, reproductive toxicity and skin sensitizatio. Foodstuff application of GCFE powders, demonstrated that microencapsulated GCFEs were more effective in delaying the oxidation of sunflower oil than free GCFEs and BHT synthetic antioxidants. In this way, microencapsulated GCFE can be recommended as a great source of antioxidants for the stabilization of food systems, especially unsaturated vegetable oils.

Keywords: chlorogenic acids, caffeine, trigonelline, microencapsulation, GCFE, computational predictions, sunflower oil.

1. Introduction

The *Coffea* gender comprising approximately seventy species, being the *Coffea arabica* commonly known as Arabica coffee, which accounts for ~60% of the world's production, and *C. canephora* var. Robusta with the remaining ~40% of the production, the most marketed worldwide (Ludwig et al., 2014).

Coffee as a complex matrix contains more than one thousand compounds with stimulant and antioxidant properties. Among the various chemicals present in coffee, chlorogenic acids (CGAs) and alkaloids such as caffeine and trigonelline have been drawing attention due to their numerous beneficial health effects, giving it an important role in the prevention of various diseases associated with oxidative stress, such as certain types of cancer, premature aging, strokes, Alzheimer's, Parkinson's, cardiovascular and neurodegenerative diseases (Barcelos et al., 2014; Tajik et al., 2017). These biopharmacological effects have been largely attributed to CGAs (Farah et al., 2008) caffeine (Bean et al., 2015), and trigonelline (Folwarczna et al., 2016) present in green coffee.

Besides preventing the presumed deleterious effects of free radicals in the human body, the effects of antioxidants in preventing the deterioration of fat and other constituents of foodstuffs has been increasing in recent years (Afshari & Sayyed-Alangi, 2017). In both cases, there is a preference for antioxidants from natural rather than synthetic sources (Abdalla & Roozen, 1999). Plant-derived antioxidants are molecules which donate electrons or hydrogen atoms (Scalbert et al, 2005). A number of studies have demonstrated that when natural antioxidants are added to food, they tend to minimize rancidity, retard the formation of toxic oxidation products, help to maintain the nutritional quality and increase shelf life of foodstuffs (Fukumoto & Mazza, 2000; Chatterjee & Bhattacharjee, 2013; Chong et al., 2015).

Whole coffee fruit, i.e. the husk, the pulp, the mucilage and the seed or "bean" inclusive, and soluble whole coffee fruit extracts is a rich source of chlorogenic acids and caffeine (Mullen et al, 2011). In agreement with Nobre et al (2011), the whole coffee fruit presents a large amount of CGA comparing with seeds. However, there are scant data comparing the content of bioactives in the fresh and processed products of coffee (Farah et al., 2008). To the detriment of this, many beneficial coffee nutrients are discarded with the fruit when coffee beans are processed, or destroyed during the roasting of the coffee beans. While there is considerable anecdotal evidence of historical consumption of the whole coffee fruit by indigenous populations in Africa and the Middle East, there is little if any published material or peer-reviewed documentation. There is an international patent claiming the use of cherry coffee as a

source of nutrients and antioxidant compounds (Miljkovic et. al., 2010), a Chinese patent claiming the fermentation process to produce wine using coffee fruit (Chinese Patent CN 1021949, cited by Miljkovic et al., 2006). In Yemen, it has been reported that coffee fruit has been boiled with spices to make a beverage called ‘‘qishr” (Beckman, 2000). The whole coffee is reported chewed as raw food (Hiembach et al., 2010), and products made from whole coffee fruit were patented and claimed to be used as an ingredient in beverage products such as nonalcoholic beverages and beverage bases, coffee, tea, milk or added to food such as soups, snack chips, candies, confections, and pastries (Drunen & Hranisayljevic, 2003)

The superior chlorogenic acids and alkaloids content in coffee fruits compared to conventional beverages needs to be considered, mainly in situations where limited caffeine intake is required. Besides, there is little data on safety of compounds found in whole green coffee fruit extract (GCFE). A recent study assessed the safety of chlorogenic acids (100 mg/kg i.p. of CGA every 24 h per 5 days) in rats, but following this protocol, CGA alone did not show any toxic effect (Alarcón-Herrera et al., 2017). Heavy consumption of caffeine is related to an increased factor risk for osteoporosis, urinary incontinency, and poorer birth and child developmental outcomes (Peacock et al., 2017). On the other hand, caffeine supplementation modulates oxidative stress by increasing the antioxidant enzymes superoxide dismutase and glutathione peroxidase *in vivo* (Barcelos et al., 2014) has been proposed as potentially protective of metabolic diseases (Nordestgaard et al., 2015). It can also work as a suppressor of tumorigenic cells preventing the development of various types of cancer (Lu et al., 2014; Peacock et al., 2017). Theoretical toxicity of caffeic acid showed very low risk prediction in all *in silico* tool employed by Salgueiro et al (2016), moreover the *in silico* biological activity spectrum indicates that caffeic acid presents five properties (mucomembranous protector, hepatoprotectant, carminative, choleretic and lipid metabolism regulator) (Salgueiro et al., 2016).

Due to the lack of concise data on the safety of green coffee whole fruit extract, this study aims at predicting the toxicological potential of isolated major compounds present in GCFE, in order to guide experimental *in vivo* assays. Besides, the objectives of the current study are to encapsulate the chlorogenic acids and caffeine rich Arabica and Robusta GCFE obtained by percolation extraction in maltodextrin and gum arabic wall materials using spray dryer. The microencapsulated GCFEs powder were then characterized in terms of encapsulation efficiency and bioactive retention. Phytochemicals properties such as hydroxycinnamics acids content, caffeine, trigonelline and theophylline content, moisture content and antioxidant activity were measured in free and microencapsulated powders. Food application was designed using the

microencapsulated Arabica and Robusta GCFE as natural antioxidants in sunflower oil, which were compared to BHT synthetic antioxidant, free GCFEs, and sunflower oil without the addition of antioxidants, on the antioxidant efficiency for a storage period of 45 days aiming at delaying the oxidation of sunflower oil.

Some studies have demonstrated the effectiveness of fruit extracts such as pomegranate and mangosteen (*Garcinia mangostana* Linn.) to prevent oxidative rancidity of sunflower oil under accelerated conditions (Iqbal et al., 2008; Chong et al., 2015). Sunflower oil is a high-quality edible oil, widely used in cooking, frying, and in the manufacturing of margarine (Sadoud et al, 2014). The sunflower oil was selected in this study due to its high use in food as it is a rich source of linoleic acid, higher degree of unsaturated fatty acids (91%) compared to other vegetable oil (Kostic et al, 2013). Furthermore, it is light in taste and appearance and has high vitamin E content compared to other vegetable oils (Sadoud et al., 2014).

2. Experimental

2.1 Material

Methanol of chromatograph grade was supplied by J.T. Backer (Phillipsburg, NJ, USA), ethanol 99.5% of analytical grade was supplied by Synth (Diadema, SP, Brazil), citric acid was supplied by Quemis (Joinvile, SC, Brazil). Ultrapure water was obtained from a Millipore system (Billerica, MA, USA), maltodextrin (MA) (DE 4 - 7), gum arabic (GA), 5-*O*-caffeoyquinic acid (95%, purity), trigonelline hydrochloride (98.5% purity), caffeic acid (98% purity) and theophylline (99% purity), *p*-anisidine (99%, purity) 2-2-diphenyl-1-pycryhidrazyl (DPPH), and butil hydroxytoluene (99%, purity) were supplied by Sigma-Aldrich (St. Louis, MO, USA), 2-thiobarbituric and 1,1,3,3-Tetraethoxypropane acid were purchased from Merck (São Paulo, SP, Brazil), the sunflower oil, containing no antioxidants, was obtained from Cargill Agrícola S.A. (São Paulo, SP, Brazil). All other chemical used in this study were analytical grade reagents supplied by Nox Solution (Maua, SP, Brazil).

2.2 Equipment and apparatus

A stainless-steel percolator with capacity of 10 L (P & L, Ltda, Curitiba-PR, Brazil) was used in the extraction process and a semi-industrial spray dryer (LABMAQ modelo MSD 1.0, Ribeirão Preto, SP, Brazil) was used to obtain the free and microencapsulated extract from green Arabica and Robusta coffee fruit. The effectiveness of antioxidant activity of free and

microencapsulated extracts added in sunflower oil was studied under drastic atmospheric conditions in a climatic chamber with relativity humidity and temperature controlled (LABMAQ modelo SL206/150, Ribeirão Preto, SP, Brazil), and a high-performance liquid chromatograph (HPLC) (Shimadzu HPLC, Kyoto, Japan) equipped with a binary pump (LC-10AD), degaser with helium (DGU-2A), automatic injection system (SIL-10A) and a diode array detector (SPD-M-10A) was used to quantify the bioactive compounds present in free and microencapsulated GCFE. The identification of chemical was carried out in a HPLC (Shimadzu HPLC, Kyoto, Japan) equipped with quaternary pump (LC-20AD), online degasser (DGU-20A5), rheodyne injection valve with a 20 mL loop, a diode array detector (Shimadzu, SPD-M20A), and a mass spectrometer detector with an ion trap analyzer and electrospray ionization (ESI) source (Bruker Daltonics, model Esquire 4000, Bremen, Germany). Chromatograph separation was performed using a Shim-pack ODS-C_{18} (5 µm, 250 x 4,6 mm, Shimadzu, Kyoto, Japão) coupled with pre-column ODS-C18 (5 µm, 4 x 3 mm, Phenomenex, Torrance, CA, USA).

2.3 Sample

The green coffee fruit Arabica (*Coffea arabica*) cultivars Oeiras MG 05 and Robusta (*Coffea canephora*) of the clone code 83, cultivated in organic cultivation system, were obtained from the Experimental Farm of Leopoldina (Minas Gerais, Brazil) belonging to the Company EPAMIG, located at 21°28'.51.38" south latitude, 42°43'17.27" west longitude and 187 meters of altitude. Both Arabica, and Robusta,. The green fruits were collected, selected, washed and then dried in a circulation air oven at 40ºC until they achieved a humidity of 20%. The pharmacognostic characterization of the sample made according to Brazilian Pharmacopeia [30] revealed a moisture content 17.55 ± 0.39 and 12.32 ± 0.41 g.100g^{-1}, total ash 5.30 ± 0.09 and 4.70 ± 0.12 g.100g^{-1}, intumescence index 2.1 ± 0.1 mL and 1.66 ± 0.15, water activity 0.7 ± 0.01 and 0.6 ± 0.0, and particle size moderately coarse < 710 µm to green Robusta and Arabica coffee powder, respectively.

The quality of sunflower oil confirmed by measurement of peroxide value (PV) (AOCS Cd 8-53), free fatty acids (FFA) (AOCS Cd 3d-63), *p*-anisidine value (p-AnV) (AOCS Cd 18-90) and thiobarbituric acid reactive substances (TBARS) assay (Ke et al., 1979) showed a PV 2.25 ± 0.06, FFA 0.12 ± 0.0005, p-AnV 2.9 ± 0.06, TBARS 0.25 ± 0.002.

2.4 Extraction and microencapsulation process

Free and microencapsulated extract from green Arabica and Robusta coffee fruit were obtained by the conditions described by Faria *et al* (2018). Briefly, dried green coffee whole fruits were crushed in a blender of high performance model OBL 10/2 (Oxy, PB, Brazil) to achieve particle-size distribution ranged from 250 to 710 µm. The powder was moistening with three parts of hydroethanolic solvent (ethanol: water 68:32, w/w) for 2 hours and then the plant material was carefully transferred and packed evenly in a steel inox percolator. The remaining solvent was added to achieve the solid to solvent proportion equal 0.9:10 (w/w), and remained in contact with the powdered material for 24 h. After that, the solvent was percolated through the powder using a flow rate of 0.2±0.05 $mL.min^{-1}$ at room temperature. The liquid extract was concentrated under vacuum in a rotary evaporator Buchi® (R-220 SE model) at 40±2ºC until it reached the content of 15% of solids.

A carrier dispersion containing 30% of solids comprised by maltodextrin (MD) and gum arabic (GA) (1:1, w/w) was added to concentrated extract (1:1, w/w), or the free fluid GCFE was dried using a laboratory scale spray dryer under the following conditions: feed flow rate 500 $mL.h^{-1}$, drying air flow 45.000 $mL.min^{-1}$, inlet temperature and outlet temperature 160 ± 2°C and 125 ± 2ºC, in agreement with Faria et al (2018). In addition, empty microparticles using MD and GA (1:1, w/w), were prepared under the same spray dryer conditions.

2.5 Chromatographic analysis of chlorogenic acids and alkaloids

Twenty milligrams of microcapsules of the free and microencapsulated GCFE were dissolved in 2 mL methanol:water:acetic acid (50:42:8 v/v/v), vortexed (1 min), kept in an ultrasonic bath for 10 min, centrifuged at 15000 x g for 15 min at 20ºC, and then filtered through a nylon filter (45 µm) according to the method described by Robert et al. (2010) with slight modifications proposed by Faria et al (2018) that used less amount of sample and a smaller time of extraction in the ultrasonic bath and in the centrifuge. Twenty microliters of the extract obtained was injected into HPLC-DAD using a mixture of 80% (v/v) 10 mM citric acid (pH 2.5) and 20% (v/v) of methanol (solvent A) and methanol (solvent B) with gradient elution as previously described by Rodrigues et al (2015). The flow rate was 1 mL min^{-1} and the column oven temperature was maintained at 30°C. Detection wavelength was set at 262, 272 and 325 nm and an external analytical curve ranging with six points from 99.8 to 1742.05 µg/mL (R^2 = 0.99, $p < 0.05$), 1.25 to 37.62 (R^2 = 0,99, $p < 0.05$), 12.63 to 429.90 (R^2 = 0,99, $p < 0.05$), 37.21

to 452.16 (R^2 = 0,99, $p < 0.05$) and 39,95 to 746,43 (R^2 = 0,99, $p < 0.05$), respectively, in triplicates were used in the quantification of 5-CQA, caffeic acid, *p*-coumaric acid, trigonelline and caffeine, respectively. The 3-CQA and 4-CQA were quantified using the molar extinction coefficient for 5-CQA as correction factor.

2.6 Moisture content (%)

The total free and microencapsulated Arabica and Robusta GCFE moisture content (MC, % w.b.) was measured from 0.5 g of sample employing a halogen lamp analyzer MB 35 (Ohaus Inc., Pine Brook, NJ, USA).

2.7 Encapsulation efficiency (EE)

The encapsulation efficiency (EE, %) was determined as the ratio of the surface 5-CQA to the total amount of 5-CQA in the microcapsules as previously described by Robert et al (2010), and was calculated according to Frascareli et al (2012).

2.8 Chlorogenic acids retention (CR)

To determine the retention of bioactives during spray-drying process, the content of chemical marker 5-CQA was measured by HPLC-DAD in the dispersion, containing the concentrated Robusta or arabic GCFE and wall materials MD and GA (1:1, w/w), before drying and after drying in the powder obtained. Chlorogenic acid retention (CR) was calculated according to Frascareli et al (2012).

2.9 DPPH scavenging activity

The ability of free and microencapsulated GCFE to scavenge DPPH (2-2-diphenyl-1-pycryhidrazyl) radical was studied according to the method reported previously by Brand-Williams *et al*. (1995) with slight modification. Briefly, five milligrams of free GCFE or fifteen milligrams of microencapsulated GCFE were dispersed in 2 mL methanol: water: acetic acid (50:42:8 v/v), agitated using a vortex mixer (1 min). Afterward, the sample was kept in an ultrasonic bath for 10 min, centrifuged at 15000 x g for 15 min at 20ºC and then filtered through a nylon filter (45 µm). The GCFE solution obtained was diluted five folds to determine the EC_{50} (concentration required to obtain a 50% antioxidant effect), and then, each dilution (0.1 mL) was added to 3.9 mL of a 6 x 10^{-5} mol.L^{-1} DPPH solution. The mixture was then mixed and left for 30 min at room temperature in the dark. The absorbance of the samples was measured at 515 nm using a Shimadzu UV-VIS Spectrophotometer UV-1800 (Chiyoda-ku, Tokyo, Japan).

The solvent mixture comprising 0.1 mL of methanol: water: acetic acid (50:42:8 *v/v*) and 3.9 mL of methanol were used as a blank. DPPH solution 6 x 10^{-5} mol.L^{-1} was used as a control, and ascorbic acid and BHT was used as a standard. The analysis was done in triplicate for standard and each extract. Antioxidant activity of each extract by DPPH method was determined by calculating the percentage of antioxidant activity using reduction of DPPH absorbance.

2.10 *In silico* toxicity prediction

Toxicity prediction procedure with 5-caffeoylquinic acid, caffeic acid, caffeine, *p*-coumaric acid and trigonelline 2D structures was performed with ACD/I-Lab (Toronto, Canada), pKCSM (Pires et al., 2015) and AdmetSAR (Cheng et al., 2012) online tools, which are based on chemical similarity criteria with well-known toxic compounds. The prediction of several toxicology parameters was estimated, and the results were expressed in ‘‘-’’ for non-detected risk and ‘‘+’’ for detected risk. In addition, the probability of adverse effects and predicted values of LD_{50} were also evaluated by ACD/I-Lab tool.

2.11 Evaluation of antioxidant activity of free and microencapsulated Robusta and Arabica GCFE in delaying the oxidation of sunflower oil

2.11.1 Experimental design

The effectiveness of free and microencapsulated Arabica and Robusta GCFE as antioxidant in foodstuff were assessed by an accelerated test in sunflower oil stored at extreme temperature and humidity, in agreement to ICH Q1F guideline (2006) applied to stability study of products marketed in Climatic Zones III and IV. Free and microencapsulated GCFE containing around 0.04% of 5-CQA, as previously assessed by Luzia et al (1997) in soy oil, was added into 60 g of sunflower oil packaged in an open transparent glass. Due to the low solubility of the free and microencapsulated GCFE in sunflower oil, it was necessary to incorporate it in ethanol. As described in a previous report (Luzia et al, 1997), ethanolic solutions were prepared with some modification in the content of solvent and time of dissolution in magnetic stirrer. Thus, different amount of microencapsulated and free GCFE weighed to achieve 0.04% of 5-CQA or BHT was dissolved in 2 mL of absolute ethanol in an ultrasonic bath during 10 min and afterwards added to the oil (60 g), the mixture was homogenized in a magnetic stirrer for 30 minutes. The same amount of absolute ethanol was used to dissolve empty microparticles to be added in sunflower oil.

The samples were stored in a climatic chamber at 40ºC ± 2ºC/75% ± 5% R.H. and were analyzed at the beginning (T_0), after 15 days (T_1), 30 days (T_2), and 45 days of storage. Synthetic antioxidant butil hydroxytoluene (BHT) at its legal limit of 0.02%, used as positive control; empty microparticles produced with GA and MD (1:1, w/w) or sunflower oil without addition of antioxidants as negative control were also stored and analyzed. Nine samples were stored for each type of sample, totaling 63 samples. At each time, 3 samples of each type were analyzed. The study was designed according to the flowchart below (Figure 1).

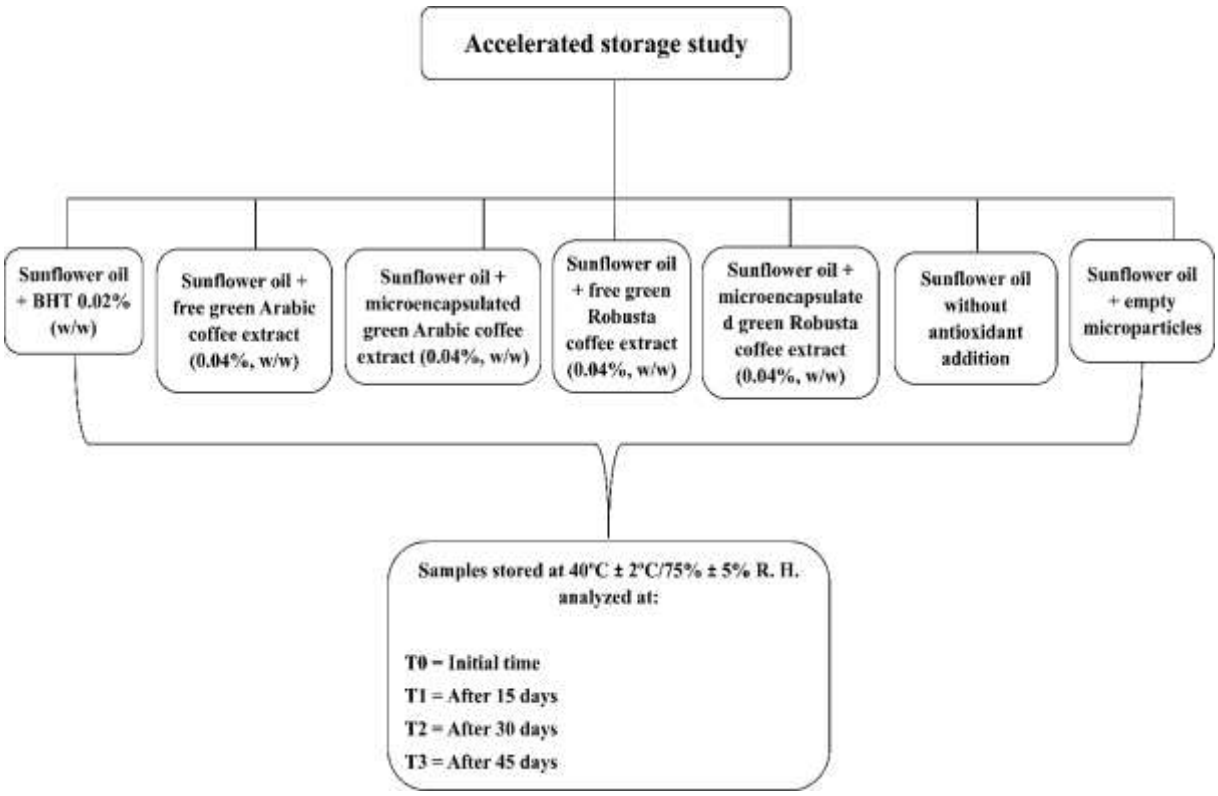


Fig 1. Systematic scheme of the antioxidant effectiveness assay of the free and microencapsulated

2.11.2 Analysis of peroxide value

Peroxide value (PV) of all samples was measured according to AOCS Cd 8-53. Sunflower oil (5±0.05g) was dissolved in 30 mL of acetic acid–chloroform (3:2; v/v) solution and stirred until the complete dissolution of sample. Then, 0.5 mL of saturated solution of potassium iodide (KI) was added and was kept in the dark for 1 min. After addition of 30 mL distilled water, the mixture was titrated against 0.01 N sodium thiosulphate ($Na_2S_2O_3$) until the yellow color disappeared. Subsequently, 0.5 mL of starch indicator (1%) was added where the titration continued until the blue color of the mixture disappeared. The blank was analyzed

under similar conditions. Determination of PV of each sample was carried out in triplicates and the results were express in meq.kg^{-1}.

2.11.3 Free fat acids (FFA)

The FFA contents of sunflower oil was determined through the titration of the sample with a standardized 0.1 mol.L^{-1} solution of KOH in isopropyl alcohol in agreement with the official AOAC method Cd 3d-63, with some modifications. Briefly, sunflower oil (2 g) was dissolved in 25 mL of ether-alcohol (2:1, v/v). After addition of drops of phenolphthalein, the mixture was titrated against 0.1 or 0.01 M KOH solution until the appearance of a slight pink color, which persisted for 30 seconds. The percentage of free fatty acids was expressed as % FFA (oleic acid) = volume mL of 0.1 or 0.01 M KOH used according to Chong et al (2015).

2.11.4 Measurement of p-anisidine value

The *p*-anisidine value (p-AnV) was determined according to AOCS method Cd18-90. To begin with, 1 g of the sample was measured directly into a 25 mL volumetric flask that was completed with isooctane solvent. The absorbance from 2.5 mL sample was measured in glass cuvettes at 350 nm against a pure isooctane blank. Then, 5 mL aliquot of this mixture was mixed with 0.5 mL 0.25% *p*-anisidine in acetic acid (w/v). The mixture was shaken vigorously and kept in the dark for 10 min, and absorbance was measured at 350nm using a spectrophotometer (Shimadzu UV-1800, Tokyo, Japan). The p-AnV was calculated according to Chong et al (2015).

2.11.5 Determination of total oxidation (TOTOX) value

The total oxidation (TOTOX) values of sunflower oils added with free or microencapsulated GCFE, BHT or control samples were determined based on the obtained PV and p-AnV values as described by Nyam et al (2013).

2.11.6 Thiobarbituric acid reactive substances (TBARS) assay

The TBARS value was determined according to the method described by Ke et al (1979). First, 10 mg of samples were accurately weighed directly into a test tube. Then, 5 mL of TBA work solution containing TBA aqueous solution (0.04 M): chloroform: Na_2SO_3 0.3 M solution (1.5:1:0.083 w/v/v) were added into the tube. The mixture was vortexed and incubated in water

bath (95 °C) for 45 min, and then cooled in cold water, 2.5 mL trichloroacetic acid (TCA) solution was added to the test tube. To separate the chloroform phase from the water phase, the samples were centrifuged at 2500 g for 10 minutes. The water phase was gently transferred from the test tube into a glass cuvette and absorbance was measured against distilled water as a reference at 538 nm. For calculations a standard curve of 0.1 mM TEP (1.1.3.3 tetraethoxypropane) working solution ranged from 0 – 35 nmol was constructed. The value of TBARS was expressed in μM TBARS. G^{-1} fat.

2.12 Statistical analysis

The data obtained were analyzed by running one-way analysis of variance (ANOVA) using GraphPad Prism 5.0 software. A one-way ANOVA and Tukey-Kramer test, with significance level of 95% ($P < 0.05$), were used to evaluate difference in the mean value of samples and controls.

3. Results and Discussion

1. Characterization of free and microencapsulated GCFE powder

The dried products showed moisture contents ranging from 1.46±0.05 to 2.33±0.1% (w/w). According to literature (USP, 2007), for pharmaceuticals powders, including spray-dried extracts, moisture content values lower than 5% (w/w) were considered adequate. In agreement with Couto et al (2013), the residual moisture is closely related to the efficiency of the drying process.

Besides temperature and feed flows, both adjusted in spray dryer apparatus, the reduced moisture content is also related with type of wall material chosen to cover the core. In accordance to Tonon et al (2008) and Ferrari et al (2012) that used the spray dryer method to dry black cherry pulp, the higher content of maltodextrin (25%) contributed to the reduction in moisture content and hygroscopicity of the material. In this study was used 50% of maltodextrin which culminated in a low moisture content in both microparticles obtained. Therefore, all free and microencapsulated powders obtained shown in table 1 presented suitable levels of residual moisture. The free and microencapsulated Arabica GCFE showed highest moisture contents than Robusta' products ($p<0.05$), it appears to be due to the larger content of sucrose in *C. arabica* (Ky et al., 2001).

Table 1. Encapsulation efficiency, chlorogenic acid retention, moisture content, and IC_{50} DPPH scavenging activity obtained for free and microencapsulated green *Coffea arabica* and *Coffea canephora* hydrophilic extract.

Sample	CR (%)	EE (%)	Moisture content (%)	IC_{50} of DPPH scavenging activity ($\mu g.mL^{-1}$)
Free Arabica GCFE	---	---	2.33±0.10[a]	4.52±0.20[a]
Microencapsulated Arabica GCFE	73.72±2.49	97.36±0.03	1.95±0.04[b]	10.81±0.70[b]
Free Robusta GCFE	---	---	2.04±0.06[b]	2.23±0.08[c]
Microencapsulated Robusta GCFE	59.61±1.30	96.90±0.04	1.46±0.05[c]	9.30±0.80[d]
Ascorbic acid				0.20±0.00[e]
BHT				2.10±0.08[b]

Results presented as ± SD mean values in columns with different letters are significantly different in Tukey's test ($p \leq 0.05$). CR = chlorogenic acids retention; EE = encapsulation efficiency; CGFE = green coffee fruit extract.

The influence of different combinations of wall materials in the encapsulation efficiency of hydrophilic extract from green Robusta coffee whole fruit was studied previously by Faria et al (2018). In their study, microparticles were prepared using different proportions of gum arabic and maltodextrin. The carrier dispersion prepared with maltodextrin: gum arabica (1:1, w/w) showed a good encapsulation efficiency, stability of GCFE, and lack of wall fissures or porosity on the particles surface indicating complete coverage of the wall material over the core that confirm the high EE values (≥98%). The rate of core to carrier dispersion ranging from 1:1 to 1:4 (w/w), does not seem to affect the EE, stability of the bioactives present in GCFE, and chlorogenic acid retention (CR). In this way, both hydrophilic extract from green *C. arabica* and *C. canephora* whole fruit, were microencapsulated using the rate of GCFE to carrier dispersion 1:1 (w/w).

In the present study, the EE achieved 96.9±0.04 and 97.3±0.03% to Arabica GCFE and Robusta GCFE encapsulation, respectively. These results are better than other studies that assessed the spray drying technique and the mixture of maltodextrin and gum arabic as wall

material. For example, Chatterjee et al (2014) achieved a spray dried microencapsulated algal extract powder with maximum EE of 65% using maltodextrin: gum arabic (80:20, w/w) as wall material. Values of the EE ranging from 11.7 to 40.5% were obtained in spray dried turmeric oleoresin with different proportions of maltodextrin and gum arabic (Cano-Higuita et al, 2015).

Due to its simplicity and low cost, the DPPH method has been widely applied for estimating antioxidant capacity and for screening new antioxidant compounds to be added in foodstuffs (Molyneux, 2004). The IC_{50} of DPPH scavenging activities in different spray-dried products of Arabica and Robusta coffee were shown in table 1. It was compared to IC_{50} of ascorbic acid and BHT standard. The lowest value of IC_{50} means had the highest antioxidant activity. In agreement to DPPH original method proposed by Blois (1958) the sample which had IC_{50} lower than 50 $\mu g.mL^{-1}$ was a very strong antioxidant, 50 – 100 $\mu g.mL^{-1}$ was strong antioxidant, 101 – 150 $\mu g.mL^{-1}$ was a medium antioxidant, while a weak antioxidant with IC_{50} > 150 $\mu g.mL^{-1}$ (Blois, 1958). Based on these values, it can be concluded that all spray-dried green coffee fruit products can be classified as very strong antioxidants.

In the present study the IC_{50} of DPPH scavenging activities of free and microencapsulated GCFE from Arabica and Robusta ranged from 2.23 to 10.81 $\mu g.mL^{-1}$. Free Robusta GCFE had the lowest IC_{50} of DPPH (2.23±0.08 $\mu g.mL^{-1}$), while IC_{50} of DPPH of ascorbic acid was 0.2±0.01 $\mu g.mL^{-1}$. On the other hand, BHT showed a strong IC_{50} of DPPH, and it was similar to free Robusta extract ($p>0.05$). In the study carried by Sentkowska et al (2016) the Robusta coffee extracts also exhibited higher capacity to scavenge DPPH radical than Arabica. This can be explained by the fact that Robusta coffee be richer in chlorogenic acids compared to Arabica coffee (Ludwig et al., 2014). Comparing different prepared green coffee, raw coffee presented a greater scavenging activity compared to steamed coffee (Sentkowska et al., 2016). DPPH assay is measured substantially water-soluble phenolic antioxidant activity so the content of phenolic compounds in vegetable extracts is directly proportional to the antioxidant power by the DPPH method (Afshari & Sayyed-Alangi, 2017).

A significant difference was observed between all samples ($p<0.05$), the free GCFEs showed a greater capacity to scavenge DPPH radical compared to microencapsulated GGEs. This could have happened due to the fact that hydroxyl, phenol, carbonyl, and aromatic amide groups present in purine alkaloids caffeine, theophylline and theobromine present in coffee are covered by the wall material which could hamper their antioxidant capacity. These results agree with Isailović et al (2012) that work with microencapsulation of natural antioxidants from *Pterospartum tridentatum* with alginate and inulin (40.7%) and observed a decrease in the percentage of DPPH scavenging capacity of the microencapsulated extract compared to free

extract (57.5%). This could be a result of the ability of wall materials in delaying the release of antioxidants encapsulated into microparticles (Dang et al., 2015). On the other hand, encapsulated natural antioxidant extracts show a higher antioxidant capacity by standard DPPH assay when added to complex matrix as foodstuffs than non-encapsulated extract (Chatterjee & Bhattacharjee, 2013).

Different plant extracts and synthetic antioxidants show different antiradical activities due to the types and content of their polyphenolic and other phytochemical constituents. Besides the hydroxyl group position, the presence of other functional groups such as double bonds and the composition of the hydroxyl groups play an important role in antioxidant activity (Memon et al, 2007). For example, to reduce the free radical DPPH, it is necessary two molecules of the amino acid lysine, which present one thiol group to the reduction of two molecules of DPPH, that is, a 1:1 stoichiometry. If, however, the molecule has two adjacent sites for hydrogen abstraction which are internally connected, as is the case with ascorbic acid (Vitamin C), the stoichiometry will be 2:1, that is, two molecules of DPPH reduced by one molecule of ascorbic acid (Molyneux, 2004).

Fidrianny et al (2016) studied regarding antioxidant capacity of various Arabica green coffee extract obtained by reflux using various polarity solvents. The lowest IC_{50} of DPPH scavenging capacity (0.7 $\mu g.mL^{-1}$) was observed for ethanolic extract and the higher was given by n-hexane extract. Coffee infusions prepared from green beans of Robusta showed a higher antioxidant capacity than Arabica extracts by the DPPH method (Jeszka-Skowron et al, 2016). These results corroborate with the values found in this present study, since a higher antioxidant activity to products from Robusta coffee was observed.

3.2 Chlorogenic acids derivatives and alkaloids presents in free and microencapsulated GCFE

A typical chromatogram of the separation of trigonelline, theophylline, caffeine and chlorogenic acid derivatives obtained for hydrophilic extract of green Arabica coffee at 272 and 325 nm is depicted in supplementary material figure 1. The contents of individual bioactives in free and microencapsulated green Arabica and Robusta coffee extracts are presented in table 2.

In free and microencapsulated green Arabica and Robusta hydrophilic extract 5-CQA was the most abundant CQA found, 61.61% in free and 64.05% in microencapsulated Arabica GCFE, and 54.15 and 60.11% in free and microencapsulated Robusta GCFE, respectively. These results agree with Farah et al (2005), since these authors described that the most abundant

bioactive in green coffee samples is the caffeoylquinic acid (CQA) derivatives, which represent about 75% of the total chlorogenic acids found in Arabica and Robusta coffee, being 5-CQA the major CQA. Besides, a higher concentration of three main 5-, 4- and 3-CQA were determined for Robusta GCFE, corroborating to Jeszka-Skowron (2016) results.

It is well established that green coffee is rich in antioxidant compounds, such as CGAs, phenolic acids, and alkaloids; and their contents depend mainly on the coffee species and their origin (Ky et al, 2001), but it is also related with degree of maturation (Koshiro et al 2007; Clifford & Kazi, 1987) and cultivation (conventional or organic agriculture) (Hecimovic et al, 2011; Carvalho et al, 2011). The total CGA in Robusta free GCFE was 38% higher than average content in free Arabica GCFE, while in microencapsulated Robusta the content of total CGA was 22.5% higher than the content measured in microencapsulated Arabica GCFE. The biggest difference between the content of total CGA in free and microencapsulated products is due to the higher retention in chlorogenic acids (CR) found in microencapsulated Arabica GCFE (73.7±2.5%) in front of microencapsulated Robusta GCFE (59.6±1.3%) as shown in table 1. Farah et al (2005) and Rodrigues et al (2015) also found a higher content of CGA (sum of 3-CQA, 4-CQA, and 5-CQA) in the hydrophilic extract of green Robusta coffee compared to a hydrophilic extract of green Arabica coffee. i.e. 5770.5±57.3/4152.9±45.1 $mg.100g^{-1}$ (Farah et al., 2005) and 25413±288/17929±409 $mg.100g^{-1}$ (Rodrigues et al., 2015) found in Robusta and Arabica green coffee hydrophilic extract, respectively.

The sum of CGAs and alkaloids caffeine, trigonelline and theophylline in Robusta GCFE contents represented 36.4% dmb on average (table 2). This species showed higher absolute contents for all, except to trigonelline that was 32 % higher in Arabica GCFE, corroborating with results found by Ky et al (2001) when they compared the GCAs, caffeine and trigonelline contents between *Coffea canephora* and *C. arabica* species.

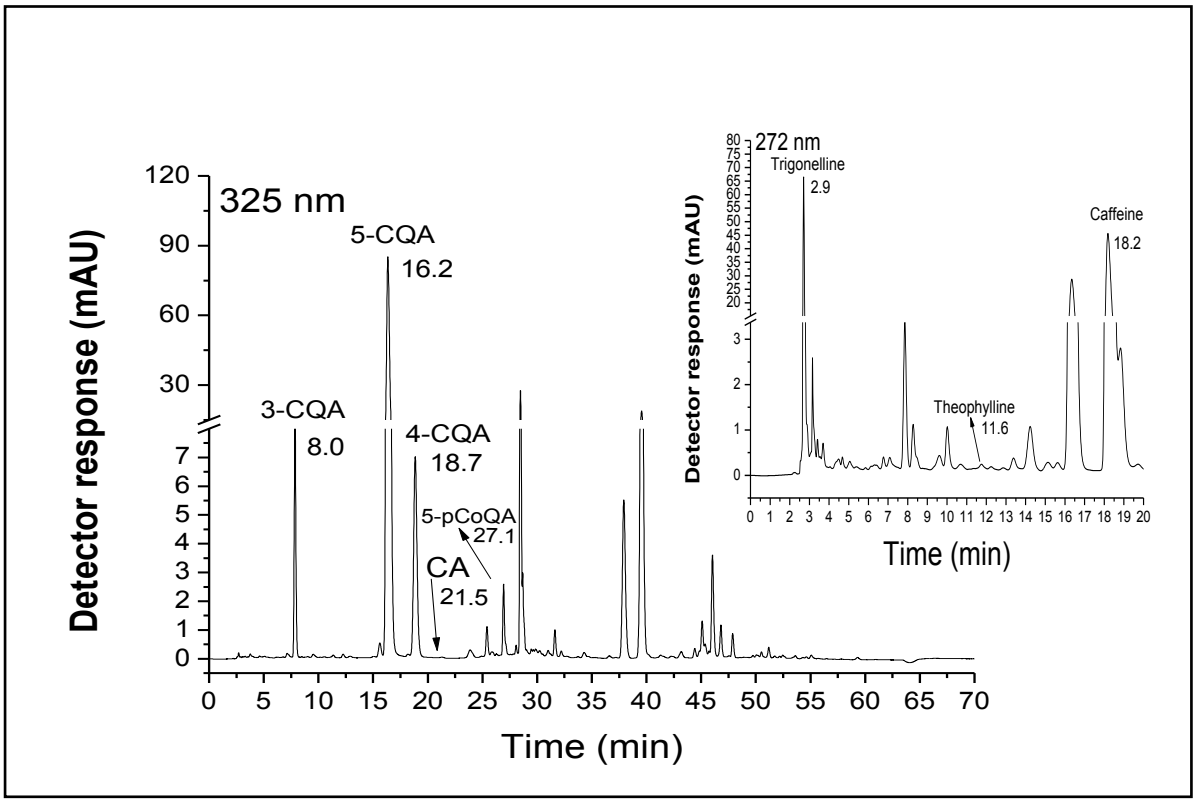


Fig 2. Chromatograms of trigonelline, theophylline, caffeine, caffeoylquinic acids (3-CQA, 4-CQA, and 5-CQA), caffeic acid (CA), and *p*-coumaric acid or 5-*p*-coumaroylquinic acid (5-pCoQA) of the spray-dried hydroethanolic extract of green Arabica coffee, obtained by HPLC-DAD recorded at 325 and 272 nm.

Table 2 – Chlorogenic acids derivatives (3-CQA, 4-CQA, 5-CQA), caffeic acid and *p*-coumaric acid and alkaloids content in free and microencapsulates extract from green Robusta and Arabica coffee whole fruit in mg/100g dm.

Sample	Hydroxycinnamic acids						Alkaloids		
	Caffeic acid	*p*-coumaric acid	3-CQA	5-CQA	4-CQA	Total CGA	Caffeine	Trigonelline	Theophylline
Free Arabica GCFE	n.d.	799±39[a]	2178±15[a]	828±126[a]	2980±49[a]	13438±190[a]	6991±78[a]	5236±68[a]	33±1.2[a]
Microencapsulated Arabica GCFE	n.d.	303±7[b]	690±15[b]	3017±60[b]	1003±62[b]	4710±137[b]	2913±33[b]	2205±4[b]	14±1.3[b]
Free Robusta GCFE	97±2.5 [a]	2245±79[c]	4842±61[c]	11753±158[c]	5108±189[c]	21703±408[c]	8746±205[c]	3557±125[c]	42±3.6[c]
Microencapsulated Robusta GCFE	21±0.1[b]	669±19[d]	1034±41[d]	3656±15[d]	1392±22[d]	6082±77[d]	3082±2[b]	1024±9[d]	11±0.5[b]

Results presented as dry mass (dm) of spray-dried free and microencapsulates extract from green Robusta and Arabica coffee whole fruit ± SD mean values in columns with different letters are significantly different in Tukey's test ($p \leq 0.05$). 3-CQA = 3-*O*-Caffeoilquinic acid, 4-CQA = 4-*O*-Caffeoilquinic acid, 5-CQA = 5-*O*-Caffeoilquinic acid, CGA = Chlorogenic acids.

3.3 *In silico* toxicity prediction

The *in silico* toxicophorical analysis was performed in order to screen the potential hazards for major compounds found in GCFE to guide the experimental assays of GCFE. Prediction data revealed no toxicological potential for most of the parameters evaluated with non-detected risk regarding AMES toxicity, carcinogenicity, hERG inhibition, binding to the estrogen receptor and skin sensitization. In contrast, only a predicted risk of hepatoxicity was attributed to caffeine (Table 3).

Although no toxicity was predicted in the AMES test, a short-term bacterial reverse mutation test, it is known that mutagenic compounds can be formed from trigonelline when green coffee is heated in roasting process (Wu et al., 1997). The compounds were also predicted as non-inhibitor of the hERG channel, whose inhibition may lead to ventricular arrhythmia (Priest et al., 2008). Caffeine and chlorogenic acids, including 5-CQA, have been positively correlated to the reduced risk of cardiovascular diseases (Cornelis et al., 2007).

A non-detected risk was predicted regarding skin sensitization, a potential adverse effect for dermally applied products (Pires et al., 2015). Furthermore, chlorogenic acid and caffeic acid present in coffee were shown to reduce chemical carcinogenesis in mouse skin (Bode et al., 2014), besides enhancing the skin wound healing in mice model (Affonso et al., 2016). The compounds were not predicted to bind to the estrogen receptor, whose binding may cause the endocrine system disruption and reproductive toxicity (Shanle et al., 2011).

On the other hand, a detected risk of hepatoxicity was predicted for caffeine (Table 3). A compound classed as hepatotoxic is likely to be associated with disrupted normal function of the liver (Pires et al., 2015). According to DiPetrillo et al (2002), in alcohol-pretreated hepatocytes, caffeine may enhance acetaminophen toxicity and the effect was attributed to activation of CYP3A.

Although no alerts of toxicity were detected for the main parameters evaluated, it does not necessarily mean that the compound will not have any adverse effects (Modi et al., 2012). Then, an additional analysis was performed with ACD/I-Lab tool in order to assess the likelihood for compounds to cause any adverse effects on a number of organs and their systems within the therapeutic dose range.

Table 3. Results of toxicity parameters for alkaloids and chlorogenic acids derivatives evaluated by prediction tools.

Toxicity parameters	5-CQA	Caffeic acid	Caffeine	*p*-coumaric acid	Trigonelline
AMES toxicity[1,2,3]	-	-	-	-	-
Carcinogenicity[2]	-	-	-	-	-
hERG inhibitor[1,2,3]	-	-	-	-	-
Hepatoxicity[3]	-	-	+	-	-
Binding to the estrogen receptor[1]	-	-	-	-	-
Skin Sensitisation[3]	-	-	-	-	-

[1]ACD/I-Lab [2]AdmetSAR [3]pKCSM

Prediction of adverse effects of compounds suggest that 5-CQA present the higher probability to cause adverse effects on blood, cardiovascular system, gastrointestinal system, kidney, liver and lungs. However, there is a general lack of information regarding these toxic effects of 5-CQA in the literature. All other compounds presented low potential to cause any adverse effects on organs and their systems evaluated (Table 4).

Although 5-CQA was predicted with higher potential to adverse effects, a comparative analysis of predicted oral LD_{50} values revealed that caffeine present lower LD_{50} when compared with other compounds, including 5-CQA (data not shown). This fact may highlight the adverse effects of caffeine in the extract, including the adverse effects reported such as hemorrhage, gastrointestinal hypermotility, acute pulmonary edema and changes in structure and function of organs (Dworzański et al., 2009).

The possibility of compounds presents adverse effects on organs and systems evaluated can contribute to better knowledge of toxicological potential of the main phytochemical identified in the extract. Moreover, the toxicological profile observed for the GCFE may be influenced by a synergistic effect of the compounds.

Table 4. Probability of adverse effects for alkaloids and chlorogenic acids derivatives using ACD/I-Lab tool.

adverse effects*	5-CQA	Caffeic acid	Caffeine	*p*-coumaric acid	Trigonelline
Blood	0.89	0.14	0.12	0.14	0.11
Cardiovascular system	0.83	0.17	0.08	0.12	0.09
Gastrointestinal system	0.97	0.06	0.27	0.04	0.28
Kidney	0.13	0.05	0.17	0.04	0.3
Liver	0.81	0.14	0.13	0.09	0.16
Lungs	0.7	0.12	0.1	0.1	0.02

*The scale of probability ranges between 0 for the lower and 1 for the higher probability.

2. Evaluation of antioxidant activity of free and microencapsulated Robusta and Arabica GCFE in delaying the oxidation of sunflower oil

Hydroperoxides are the main primary oxidation products, accumulating during the initiation and propagation step of the oxidation process (Fennema et al., 2007). According to Frankel (2005), the level of hydroperoxide in the oxidation process is related to the number of double bonds, and it occurs earlier in highly unsaturated lipid because their hydroperoxide decomposes more easily. The degree of primary oxidation of sunflower oil was determined by measuring PV in the presence or absence of free and microencapsulated GCFE as potential antioxidants, or in the presence of synthetic antioxidant under accelerated condition (40ºC ± 2ºC/75% ± 5% R. H) for 45 days.

As it can be seen in Figure 3, the PVs of all treatments increased over the time. After 15 days of storage, the sample without addition of antioxidant (only sunflower oil, negative control) or added to empty microparticles showed a PV significantly higher than others added of antioxidant ($p<0.05$), except to positive control which was not significantly different to the controls ($p>0.05$). After 30 days, the free Arabica GCFE presented a lower PV compared to the

other samples ($p<0.05$). But at the end of the accelerated study, the sunflower oil containing free Arabica and Robusta GCFE showed a similar increase in PV ($p<0.05$), while the sample containing microencapsulated Arabica GCFE had a lower increase in PV ($P<0.05$). The PV results of the sample containing microencapsulated Robusta GCFE and BHT were similar ($p>0.05$), both showed a higher PV compared to the samples without antioxidant, empty microparticles and free Arabica and Robusta GCFE ($p>0.05$). Despite of the antioxidant effectiviness in food matrix, the safety of synthetic antioxidant BHT is aways a controversial discussion because of their possible toxic effects during long-term intake (Taghvaei & Jafari, 2014). According to the study of Lanigan and Yamarik (2002) the BHT showed a potential carcinogenic effect on liver, kidney and lungs of rats. Thus, reduce or replace synthetic antioxidant by natural antioxidants that are more compatible with human nature, is a logical choice.

The sunflower containing microencapsulated Arabica presented a lower PV value after 45 days of storage compared to all the other samples ($p<0.05$). Although some studies have shown the antioxidant effect of GA and/or MD (Kadman et al., 2017; Rodrigues et al., 2012; Faria et al., 2010), in this study it was not observed any effect of this biopolymers in delaying the lipid peroxidation.

According to the Codex Standard for Named Vegetable Oils, the PV in refined oil should be less than 20 meq/kg oil, and the data show that in the initial time (T0) PVs were lower than this (2.25±0.005 – 2.26±0.002 meq/kg oil). However, after 15 days all samples achieved PV between 28.6±1.3 and 37.6±1.3 meq/kg oil. And in the end of the study, the PV of all treatments were higher than 100 meq/kg oil.

Sadoubi et al (2014) studied the thermo-oxidative profile of sunflower oil, without antioxidant addition, applying heating at 99±2°C with incorporation of 9 L of oxygen/second for 52 h continuously and found that PV 26 folds higher in thermoxidized oil compared to fresh oil. In the present study, the fresh sunflower oil showed that PV 14.7 folds lower than sunflower oil stored under 40ºC ± 2ºC/75% ± 5% R.H for 45 days. While other treatments showed an increase in PV in 14.3, 14.1, 12.02, 11.9 and 11.2 folds to free Arabica GCFE, free conilon GCFE, microencapsulated Robusta GCFE, microencapsulated Arabica GCFE, and BHT, respectively.

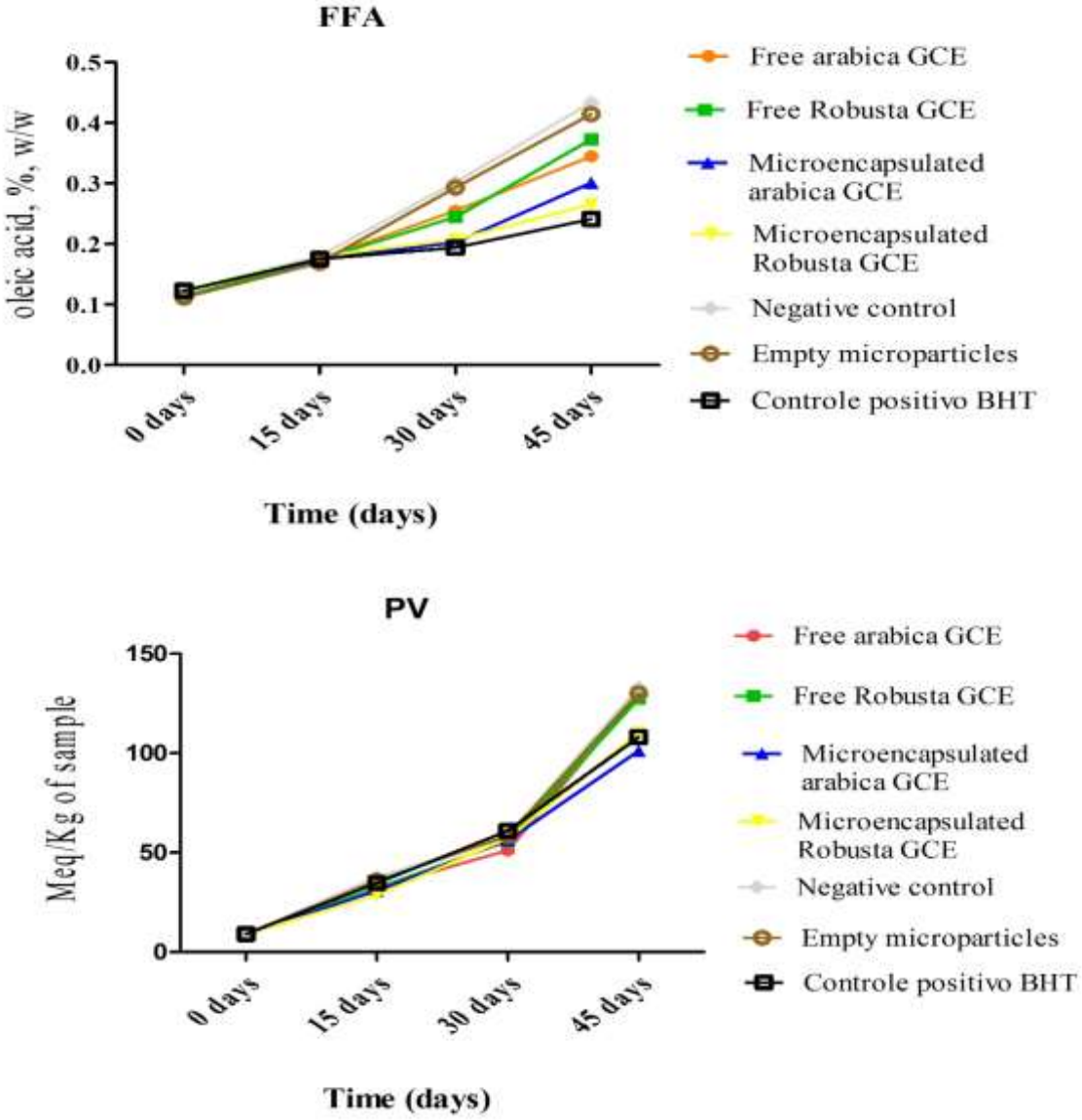


Fig 3. Free fat acids (FFA) and peroxide value (PV) changes in sunflower oil containing free or microencapsulated Arabica or Robusta GCFE or empty microparticles or BHT during 45 days at 40ºC ± 2ºC/75% ± 5% R. H

In the study carried out by Afshari and Sayyed-Alangi (2017), the authors tested the antioxidant effect of leaf extracts from Cressa cretica in soybean oil incubated for 16 days at 63°C, samples containing higher concentrations of the extract had more inhibitory effect against oxidation due to more phenolic compounds present. On the other hand, the 5-CQA applied on the concentration of 0.04% appeared to be more effective in lowering peroxide accumulation and thus improving the oxidative stability of the soya oil when compared to soya oil enriched with 0.08% of 5-CQA (Luzia et al., 1997).

In the present study, all treatments had the content of phenolic compounds standardized to be equal to each other (0.04% 5-CQA). However, the microencapsulateds systems showed similar antioxidant activities against lipid peroxidation compared to BHT, and a better antioxidant power compared to free GCFEs. It could be attributed to the fact that the controlled release of antioxidants from microencapsulated GCFEs could have possibly prevented the synergistic effect of pro and antioxidant activities, since chlorogenic and caffeic acids have been described as pro-oxidant on their redox status (Upadhyay et al., 2013) or in the presence of free transition metal ions since these compounds stimulate the formation of hydroxyl radical in the Fenton reaction (Yamanaka et al., 1997). The CGA-phenoxyl radical performed is available to induced lipid peroxidation (Liang & Kitts, 2016).

Determination of acidity provides important data on the conservation status of oil since the decomposition process almost alters the concentration of the hydrogen ions and increases the free fatty acids (FFA) formation by triglycerides decomposition. In this study, the FFA among treatments (Figure 2) showed no significant difference ($p<0.05$) during the first 15 days under accelerated condition. However, the increase in FFA formation after 30 and 45 days followed the oxidation profile observed in PVs. The FFA of all samples were significantly lower compared to samples containing empty microparticles or without antioxidant addition ($p>0.05$). This means that all the antioxidants assessed were effective in delaying the decomposition of sunflower oil. After 30 days of storage, the BHT was similar to microencapsulateds Arabica and Robusta GCFEs to delay FFA formation ($p<0.05$). After 45 days, the BHT showed higher effectiveness compared to microencapsulated Arabica GCFE ($p>0.05$), but not compared to microencapsulated Robusta GCFE ($p<0.05$). Both, microencapsulated Arabica and Robusta GCFEs, showed similar effectiveness to delay sunflower oil decomposition during 45 days of storage ($p<0.05$). Meanwhile, free Arabica and Robusta GCFE were less effective compared to BHT or microencapsulated extracts, but still they presented significant efficacy in sunflower oil compared to negative control after 45 days of accelerated study. The total increases of FFA were in the following sequence: negative control/ empty microparticles > free Robusta GCFE > free Arabica GCFE > microencapsulated Arabica GCFE / microencapsulated Robusta GCFE / BHT upon 45 days of accelerated conditions (Figure 2).

The antioxidant capacity of CGAs are related to their potential of inhibiting reactive oxygen species (ROS) or by scavenging them (Upadhyay et al., 2013). Besides CGAs and their derivatives, caffeine also reduces oxidative stress and protects antioxidant system by synergistic effect (Jeszka & Skowron et al., 2016). The 5-CQA, according to conventional theories is a

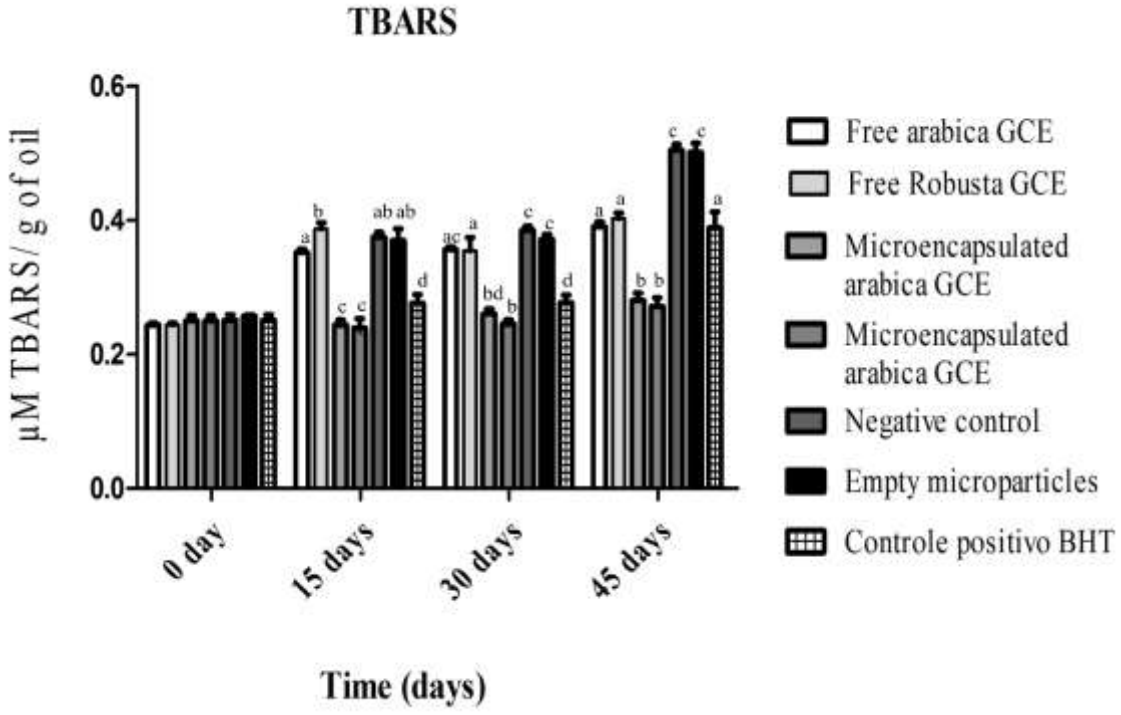
primary antioxidant due to the presence of electron-donating groups (hydroxyl) as substituents in benzene ring. In agreement to Luzia et al (1997), the 5-CQA can act rapidly with free radicals in edible oil to form radicals stabilized by electron dislocation around the aromatic ring. The main advantages of the addition of a primary antioxidant in edible oil was the extension of the induction period, through delaying the accumulation of free radicals and thus improving the stability during storage (Luzia et al., 1997). The antioxidant capacity activity of coffee is totally dependent on diversities of bioactives present in the extract since they show different antioxidant characteristic, especially CGAs, which possess *in vitro* and *in vivo* antioxidant capacity (Shahidi & Chandrasekara, 2010), by contrast, the caffeic acid that belongs to a small class of polyhydroxyl phenolics and is the simpler p-coumaric derivative, shows a relatively stronger pro-oxidant capacity than CGA that is esterified and contains a quinic-carbohydrate moiety (Liang & Kitts, 2016). Green coffee constituents, such as CGAs, caffeine and other methylxanthines, such as theophylline, show antioxidant capacity by metal chelating activity (Homma & Murata, 1995; Daglia et al., 2000). Hence, the combination of these bioactive compounds present in GCFEs might provide synergistic effects to the antioxidant capacity that further enhanced the oxidative stability of oils.

The *p*-anisidine (p-AnV) is based on the reaction between *p*-anisidine and the secondary lipid oxidation products performed when the hydroperoxide decomposes to carbonyl, ketones, and aldehydic compounds, mainly 2-alkenals and 2,4 alkadienals (O'Keefe and Pike, 2010). The p-AnV is expressed as the absorbance of solution produced from the mixture of oil in isooctane solution with *p*-anisidine reagent diluted in glacial acetic acid (O'Keefe and Pike, 2010). Among the treatments, p-AnVs were not significantly different ($p>0.05$) neither in their initial time (T0) nor after 45 days of accelerated storage (Figure 3). The p-AnV of all the treatment remained constant during the accelerated storage period, meaning that a low content of secondary products of oxidation was detected for p-anisidine reagent in all treatments. In the study of antioxidant capacity of mangosteen peel extracts in sunflower oil carried out by Chon et al (2015), after 24 days of sunflower oil storage (65ºC), the p-AnVs of all the treatment were in the range of 12.06±0.03 to 12.24±0.02, as the sunflower oil without any antioxidant had the highest p-AnV and the sunflower oil with 200 ppm of extract had the lowest p-AnV.

Free and microencapsulated GCFEs showed the same profile in p-AnV until the thirteenth day ($p>0.05$). In this time, BHT, empty microparticles, and sample without addition of any antioxidant (negative control) had the highest value of p-AnV compared to other

treatments ($p<0.05$). At the end of the accelerated storage period, the sunflower oil without addition of antioxidant showed a highest p-AnV, but it was not significant ($p>0.05$).

The oxidative stability of sunflower oil was monitored under different conditions (open flask, caped flask, and flask under N_2 atmosphere) at 47ºC (Crapiste et al., 1999). The p-AnV of the sunflower oil in open flask was 3.28 after 24 days and 16.2 after 50 days of storage, whereas in the caped flask the p-AnV was 2.75 after 30 days and 14.7 after 50 days of storage. No significant oxidation was observed in samples stored under nitrogen atmosphere (14.5 after 101 days)). In the present study, all samples were stored in an open transparent glass. When the oxygen supply is practically unlimited, as in open flasks or with high air-to-oil ratios, the oxidation rate depends on the relation between the oil surface area exposed to air and the sample volume (Crapiste et al., 1999). Bearing that in mind, all samples in this study were weighed to 60g and were added into glasses of the same dimensions.



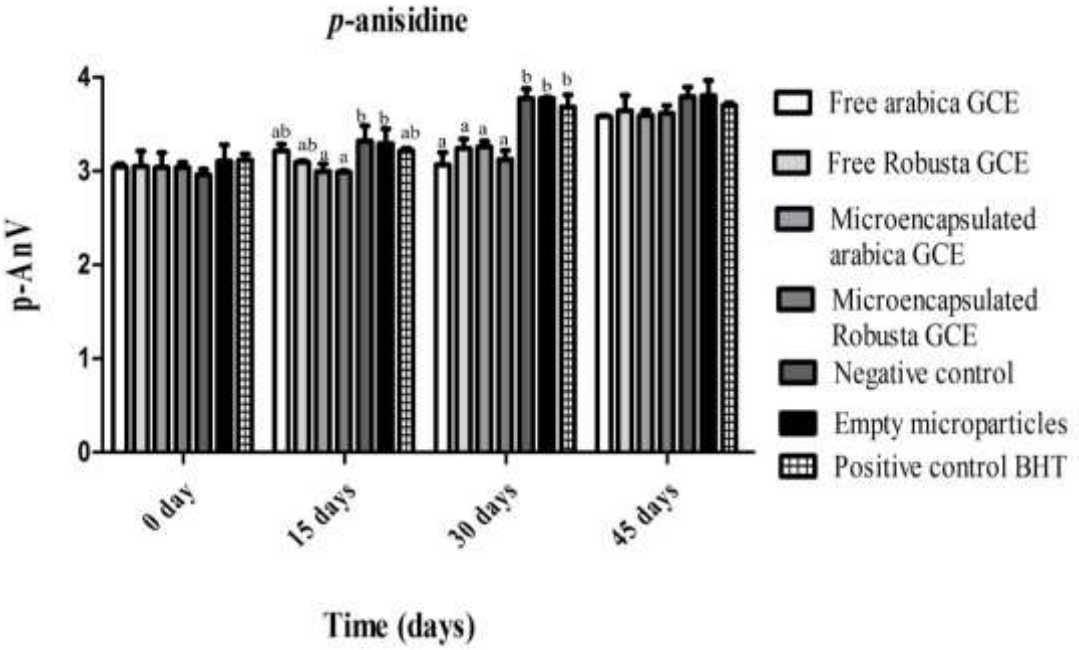


Fig 3. TBARS and *p*-anisidine values in sunflower oil containing free or microencapsulated Arabica or Robusta GCFE or empty microparticles or BHT during 45 days at 40ºC ± 2ºC/75% ± 5% R. H. Different letters within each storage period denote that they are significantly different ($p>0.05$).

The thiobarbituric acid (TBA) test is one of the oldest methods used to detect lipid oxidation and it was first proposed in the 1980's (Shahidi & Wanasundara, 2002). This method is based on the formation of a pink color complex with strong absorbance at 532 – 535 nm when TBA reacts with the oxidation products of the polyunsaturated oil (Shahidi & Wanasundara, 2002). Thus, malonaldehyd, a major secondary by-product of lipid oxidation in food samples, is the main target of TBA reactions (Afshari & Sayyed-Alangi, 2017). Based on this fact, the test is still standardized by using malonaldehyd generated from 1,1,3,3-tetraethoxypropane by acid hydrolysis (Frankel, 2005).

Figure 3 shows the TBARS values in sunflower oil containing free or microencapsulated Arabica or Robusta GCFE or BHT during 45 days at 40ºC ± 2ºC/75% ± 5% R. H. After 15 days of storage, the supplementation of sunflower oil with microcapsules containing different GCFE was significantly effective in delaying the deterioration of the sunflower oil ($p<0.05$). This was more effective than BHT of all accelerated storage periods ($p<0.05$). This result is also in accordance with the one reported by Rafiee et al (2012) who demonstrated the higher effectiveness of olive leaf extracts in front of BHA and BHT.

After 30 days of storage, both Arabica and Robusta free extracts showed the highest TBARS value compared to all treatments ($p<0.05$), except to negative control and sample added

to the empty microparticles that were not statistically different. Although GA demonstrates efficiency in reducing renal malonaldehyde in mice (Al-Majed et al., 2003) and in human blood (Kaddam et al., 2017), the amino acids tyrosine, histidine and methionine seem to be responsible for the antioxidant capacity of GA against ROS and RNS (Rodrigues et al., 2012). But in this *in vitro* assay, the GA in empty microparticle did not show any decrease in the TBARS value in sunflower oil.

After 45 days, the BHT increases the TBARS value and achieved the similar effectiveness of free Arabica and Robusta GCFE in delaying the deterioration of sunflower oil corroborating with results found by Chon et al (2015) who presented the similar TBARS value of mangosteen peel extract (200 ppm) compared to BHA synthetic antioxidant in sunflower oil.

The encapsulated Arabica and Robusta extracts compared to free extracts were more effective in delaying the oil deterioration from the first 15 days of storage until the last day of the assay. In agreement with Taghvaei and Jafari (2014), the encapsulation process acts by protecting the natural antioxidants against the destructive factors of encapsulation. The olive leaf extract encapsulated by Arabic gum and maltodextrin, added in soybean oil, showed the most oxidative protection activity (more than BHT at 100 and 200 ppm) in study proposed by Taghvaei an Jafari (2014). It has been proved that encapsulation could improve the stability of natural antioxidants (Taghvaei & Jafari, 2014).

Different to what was observed in p-AnV, the results of TBARS in different treatments were not constant as can be seen in Figure 3. TBARS test is applied to detect oxidative deterioration of the oil samples while the p-AnV test was done to measure the content of aldehydes (Winne Sia et al., 2014). In terms of sensitivity and specificity, TBARS is sensitive, not only specifically to aldehydes since TBA also reacts to ketones, ketosteroids, acids, esters, proteins, sucrose, urea, pyridines, and pyrimidines (Jardine et al., 2002). Thus, it is expected to find difference between the two tests.

The concept of total oxidation (TOTOX) value was first proposed by Holm (1972) who suggested a combined expression of peroxide and secondary oxidation products. In accordance to Holm (1972), an increase of one PV unit corresponds to an increase of two p-AnV units. This happens because during the initial phase of the lipid oxidation, the hydroperoxides may steadily increase to a maximum, then they start to decrease. At the same time, as hydroperoxides react further, aldehydes begin to form. Nyam et al (2013) noticed that the TOTOX value increases linearly during storage of the sunflower oils supplemented with various vegetable extracts. In an assessment of the antioxidant capacity of grape seed extract in sunflower oil, the TOTOX value was found to be one of the best indicators of fat oxidation (Poiana, 2012).

To be considered a good quality oil, the TOTOX value must be less than 10 (Hamilton et al., 1983). In the present study, all treatments showed a TOTOX value below 10 at initial time ranged from 7.49±0.04 to 7.61±0.06. The TOTOX value of all treatments increased linearly during storage, and after 45 days of the accelerated storage, the TOTOX value of all samples ranged from 206.05 ± 0.2 to 269.57 ±2.5. Thus, the microencapsulated Arabica GCFE showed the lowest and negative control whereas the empty microparticles showed the highest TOTOX value after 45 days of storage ($p<0.05$), see Figure 4.

The TOTOX values for samples mixed with microencapsulated Arabica and Robusta GCFE were significantly lower than the value registered for all other treatments ($p<0.05$) after 45 days. The BHT showed a similar TOTOX value with the microencapsulated Arabica GCFE ($p>0.05$), this synthetic antioxidant showed to be more effective in delaying the oxidative process in sunflower oil compared to negative control, the empty microparticles and to all the free GCFEs ($p<0.05$).

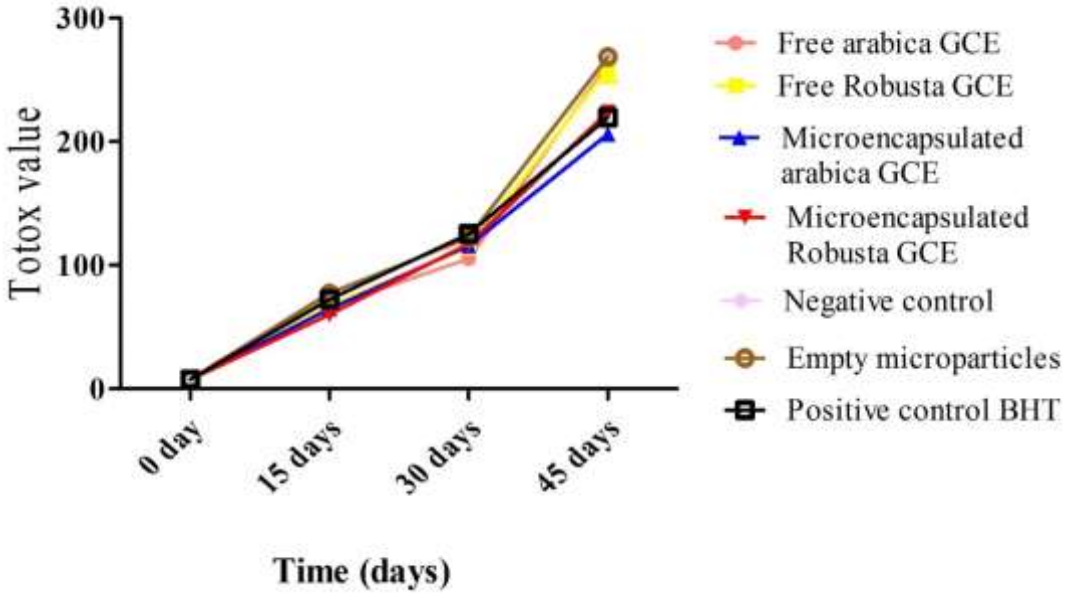


Fig 4. TOTOX value in sunflower oil containing free or microencapsulated Arabica or Robusta GCFE or empty microparticles or BHT during 45 days at 40ºC ± 2ºC/75% ± 5% R. H

4. Conclusion

The extraction and microencapsulation process were successfully applied to green coffee whole fruit extracts since a high content of bioactives from *Coffea arabica* and *Coffea canephora* was obtained. In addition, a good encapsulation efficiency was achieved. The

antioxidant capacity assessed by DPPH assay showed that the encapsulation process decreased the antioxidant capacity of both GCFE. This could be attributed to the controlled release of antioxidants from the encapsulated extracts. The *in silico* toxicity performance of most chemical compounds found in GCFE revealed no toxicological potential for most of the parameters assessed, including carcinogenicity, hepatotoxicity, and skin sensitization. Using ACD/I-Lab tool, the 5-CQA was predicted to cause more adverse effects than caffeine although there is little evidence on the safety of CGAs in the literature. However, it is worth mentioning that the *in silico* findings can be complementary, but they do not replace the *in vitro* and *in vivo* analysis. Foodstuff application of GCFE powders demonstrated that GCFEs microencapsulated by Arabic gum and maltodextrin standardized in 0.04% of 5-CQA, were effective in delaying the oxidation and deterioration of sunflower oil during accelerated storage conditions and demonstrated stabilizing efficacy comparable to the commonly used synthetic antioxidants, BHT (0.02%). The microencapsulated GCFE delayed significantly the lipid peroxidation and deterioration of oil by performing secondary oxidation products when compared to BTH and free extracts. This can be due to the fact that the encapsulation allowed controlled release of the antioxidant and inhibited the pro-oxidant activity of free GCFEs. In conclusion, both microencapsulated extracts can be recommended as great sources of antioxidants for the stabilization of food systems, especially vegetable oils with high amount of polyunsatured fatty acids.

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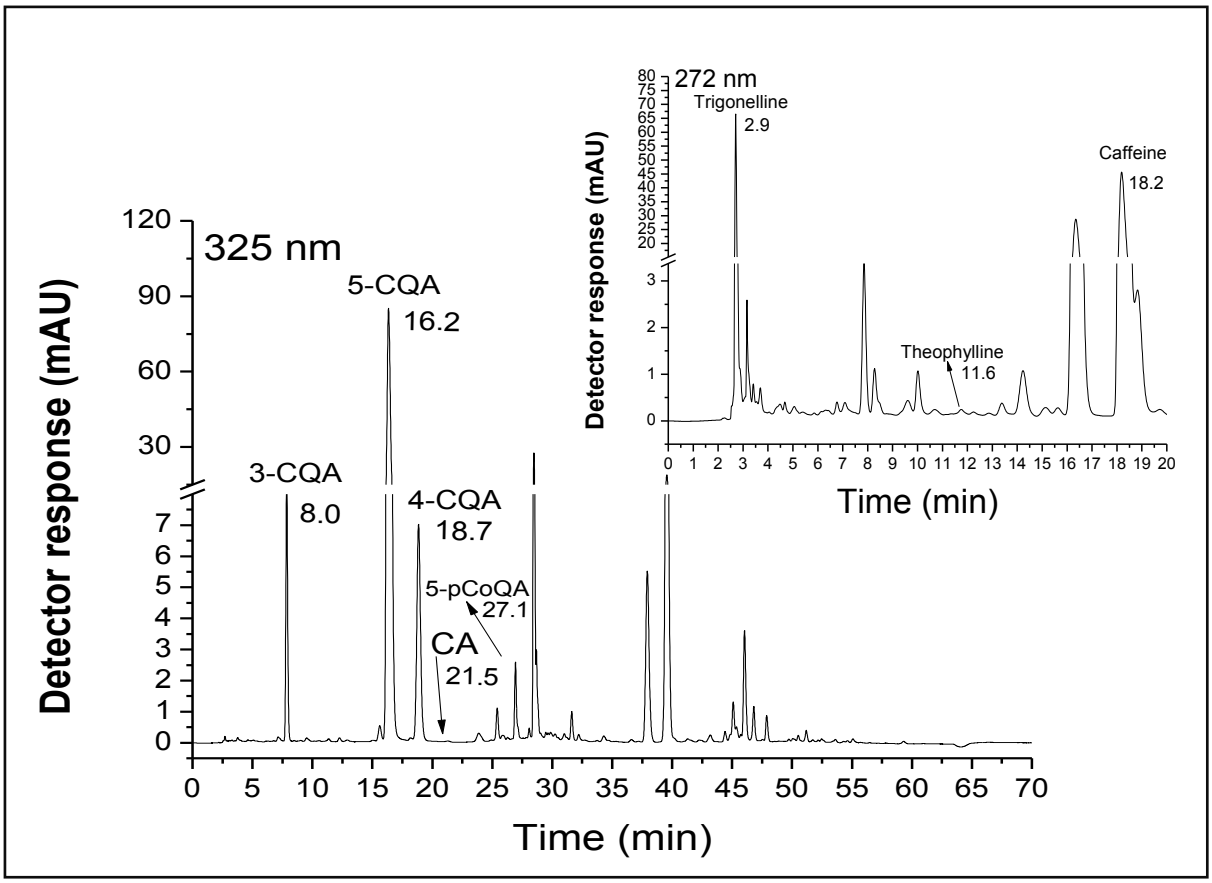
Supplementary Material



Supplementary Figure 1 1. Chromatograms of trigonelline, theophylline, caffeine, caffeoylquinic acids (3-CQA, 4-CQA, and 5-CQA), caffeic acid (CA), and *p*-coumaric acid or 5-*p*-Coumaroylquinic acid (5-pCoQA) of the spray-dried hydroethanolic extract of green Arabica coffee, obtained by HPLC-DAD recorded at 325 and 272 nm.

Table S1. Chromatographic and mass spectroscopic characteristics of the CGA and derivatives and xanthines identified in the spray dried microencapsulated GCFE as obtained by HPLC-DAD-MS.

T_R (min)	Compound	Abbreviation	λ_{max} (nm)	$[M - H]^-$ (*m/z*)	$[M + H]^+$ (*m/z*)	Fragments (*m/z*)
2,9	Trigonelline	Trig	265		138	MS^2 [138]: **121**, 110, 142, 94
8.0	3-caffeoylquinic acid	3-CQA	239, 326	353		MS^2 [353]: **191**, 179, 135, 173
						MS^3 [353 → 191]: **127**, 93, 173, 111, 81
11.6	Theophylline	Theoph	274		181	MS^2 [181]: **163**, 190, 123, 137
16.2	5-caffeoylquinic acid	5-CQA	238, 326	353		MS^2 [353]: **191**, 179
						MS^3 [353 → 191]: **127**, 173, 93, 111, 144
18.7	4-caffeoylquinic acid	4-CQA	239, 326	353		MS^2 [353]: **173**, 179, 191, 135
						MS^3 [353 → 173]: **93**, 111, 155, 71
19,8	Caffeine	Caffein	274		195	MS^2 [195]: **163**, 137, 109
21.5	Caffeic acid	CA	236, 322	179		MS^2 [179]: **135,** 119
27.1	5-*p*-Coumaroylquinic acid	5-pCoQA	234, 312	337		MS^2 [337]: **191**, 163, 173

CAPITULO VI

Acute and subacute oral toxicity assessment of the encapsulated and non-encapsulated dried extract from green coffee fruit rich in caffeine and chlorogenic acids

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ABSTRACT

The coffee fruit is a high source of bioactive compounds as phenolic acids and methylxanthines, comprising chlorogenic acids and caffeine, respectively. Extract obtained from this matrix could be used as a supplement to functional foods, energy drinks, additives or even as an active ingredient in cosmetic or pharmaceutical industry. The safety of encapsulated and non-encapsulated hydroethanolic extract from green coffee fruit (GCFE), rich in caffeine and chlorogenic acids, was assessed by acute and subacute toxicity tests. In the acute test, the oral single doses until 1000 mg/kg bw did not show any adverse effect in both female and male mice observed by Hippocratic screening and by clinical parameters for a period of 14 days. However, LD_{50} of non-encapsulated GCFE was found at a dosage of 5000 mg/kg bw, and LD_{50} of encapsulated GCFE was not achieved in this study, probably due to the release delay of caffeine and other compounds from GCFE. The stimulant effect of non-encapsulated GCFE was seen at a dosage of 1000 mg/kg bw after 30 min of oral administration, but not after 60 min. The daily consumption of encapsulated GCFE for 30 days showed no adverse effect in male rats, even at the highest dose, equal to 1000 mg/kg bw. This result indicates that no-observed-adverse-effect level (NOAEL) of encapsulated GCFE is established to be 1000 mg/kg bw/day, and, extrapolating this value for human consumption, the human equivalent dose (HED) is 189 mg/kg bw/day or 11.34 grams/day, considering an adult of 60 kg of body weight mass.

Key-words: safety, mice, rats, *in vivo*, 5-CQA, trigonelline, LD_{50}, NOAEL.

1. Introduction

Although efficacy of any medicine, food component, food additive, dietary supplement, chemical, or substances are important, safety is of paramount importance. The safety of a substance, including encapsulated products, prior to use in humans, can be determined by use of *in vitro* tests, *in silico* studies and studies in animals, especially in rodents and also in other species (Wandrey et al., 2010; Adamson, 2016). It must be considered because a range of information is required by the regulatory agencies for a petition for use of a new food ingredient, for example, in order to determine the estimated daily intake (EDI), acceptable daily intake (ADI), no-observed-adverse-effect-level (NOAEL), etc. (Gaonkar et al., 2014).

The microencapsulation of food additives and supplements, besides protecting the core substances of external environment, is a technique applied to prevent the interaction between core to food matrix nutrients and also to provide a controlled release of compounds from core (Singh et al., 2010; Sobrinho & Farias, 2012). The development of nano/microencapsulation systems that can carry, protect and deliver food ingredients has been a key breakthrough in the food industry (European Commission, 2015). However, the development of oral controlled release system is yet a challenge nowadays due to the inability to restrain and localize the system at targeted areas of gastrointestinal tract (Singh et al., 2010).

A number of research has been developed aiming to improve the safety of substances using different techniques of microencapsulation (Ferenz et al., 2014; Jarrar et al., 2017; Tzankova et al., 2017). Safety is an important concern with any new form of bioactive compounds release system. Being biocompatible, non-toxic and biodegradable, for instance, are characteristics requested for carrier materials (Alvaréz et al., 2011; Yilmaz et al., 2015). Because of biodegradability, excellent biocompatibility and ability to provide controlled-drug release natural polymers such as maltodextrin (MD) and gum arabic (GA) are often used for microsphere preparation (Yilamaz et al., 2015). The Joint Expert Committee, from the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) on Food Additives (JECFA) provide online documents about the acceptable daily intake (ADI) of substances applied in encapsulation technologies based on animal toxicology or human studies; expressed as numerical value (mg/kg bw/day – body weight per day). The ADI of gum arabic and maltodextrin is not specified, meaning that these substances have very low toxicity. While other polymers such as polypropylene glycol alginate (ADI = 70 mg/kg) and beta cyclodextrin (ADI= 5 mg/kg) already shows limited daily intake (Codex Alimentarium, 2008; Wandrey et al., 2010).

Due to their safety and cost-effectiveness, the GA and MD (DE<20) were chosen as wall material for to encapsulate hydroethanolic extract from whole coffee fruit (*Coffea canephora*). The extract obtained previously by Faria et al (2018a) shows a high content of caffeine and chlorogenic acids and it is claimed to be used as food additive and/or nutritional supplement in foodstuff. The health benefits of green coffee extract are already well documented (Onakpoya et al., 2011, Vinson et al., 2012; Ludwig et al., 2014). The efficacy of crude coffee beans extracts as antidiabetic (Lecoultre et al., 2014), anti-obesogenic (Onakpoya et al., 2011; Flanagan et al., 2014), antioxidant (Yoshida et al., 2008) and also antihypertensive (Revuelta-Iniesta & Al-Dujaili, 2014) has shown a positive result through clinical studies. But the safety of isolated compounds as chlorogenic acid (Zieger & Tice, 1998; Soga et al., 2013) and, especially, caffeine still shows divergent concerns (Barcelos et al., 2014; Hashimoto et al., 2016; Peacock et al., 2017).

There is a published study carried out by Heimbach et al. (2010) in which they assessed the safety of daily ingestion in diet (for 90 days) of ethanolic extract from whole cherry coffee fruit in rats (approximately 3446 and 4087 mg/kg bw/day) and no adverse effects were observed during the study. However, according to the international patent WO 2004/098320, this extract obtained from coffee cherry, Coffeeberry® Energy Brand, provides a lower average content of chlorogenic acids (0.3 – 14 %) and caffeine (0.0 – 4.46%) (Miljkovic et al., 2010) than the extract obtained in the present study (table 1). Besides, the Coffeeberry® is a non-encapsulated extract of coffee cherry and one of the objectives of this study was to test the safety of encapsulated form of extract from whole green coffee fruit (GCFE).

The present study was developed looking forward to establishing the acute safety, acute effect on behavior and locomotor activity, and the LD_{50} (lethal dose, 50%) of encapsulated and non-encapsulated GCFE in mice; and also, to determine the NOAEL (no-observed-adverse-effect-level) of encapsulated GCFE by subacute 30-day study in rats, since preclinical studies are a vital first step to assess the safety before human consumption.

2. Materials and Methods

2.1 Materials

The powder extracts tested by acute and subacute toxicity methods were derived from the green fruits (GCFE) of *Coffea canephora* obtained by organic culture of the Experimental Farm of Leopoldina (Minas Gerais, Brazil) belonging to the Company EPAMIG. The extraction and microencapsulation process were described by Faria et al. (2018a), and the physical-chemical characteristics of the powders tested in the following study are shown in table 1 (Faria et al., 2018a; Faria et al., 2018c).

The chlorogenic acid 5-*O*-Caffeoylquinic acid (5-CQA) was one compound found in most of the powders followed by alkaloid caffeine. Besides this one, caffeic acid, trigonelline, theophylline and *p*-coumaric acid were also identified (Faria et al., 2018b). The shelf-life of these compounds were established in previous studies by accelerated stability study for six months (Faria et al., 2018a; Faria et al., 2018b) which assure the stability of the test material during the toxicity assays.

Table 1. Physical-chemical characteristics of the encapsulated and non-encapsulated GCFE (Faria et al., 2018a; Faria et al., 2018c)

	Encapsulated powder	Non-encapsulated powder
Appearance	Pale yellow powder	Greenish/brown powder
Extraction solvent	Ethanol: water (68%, w/w)	Ethanol: water (68%, w/w)
Moisture content (%)	1.65±0.06	2.04±0.06
Water activity	0.10±0.08	0.70±0.01
Particle size distribution (μm)	1.19 – 12.65	0.88 – 4.60
Total chlorogenic acids (%)*	6.08±0.07	21.70±0.40
Caffeine (%)	3.08±0.01	8.75±0.20
Trigonelline (%)	1.02±0.08	3.55±0.12
Antioxidant capacity**	9.30±0.8	2.23±0.08

*3-*O*-caffeoylquinic acid, 4-*O*-caffeoylquinic and 5-*O*-caffeoylquinic acid.

**Measured by DPPH method (IC_{50} of DPPH scavenging activity ($\mu g.mL^{-1}$).

(%) based on approximate value. Other values were given as mean ± SD.

The powders' dosage of encapsulated (En-GCFE) and non-encapsulated (Non-En-GCFE) were equated by using 5-CQA as the chemical marker. Correction factor (fc) of 3.5 was applied for adjustment of the test dosages since one part of the non-encapsulated GCFE is equivalent to around 3.5 folds of encapsulated GCFE mass.

2.2 Toxicity study methods

The toxicological studies carried out were acute oral toxicity (OECD Guideline for testing of chemicals, OECD, Paris, 2002) and subacute toxicity that were performed following the Guideline 407 for repeated dose 28-day oral toxicity study in rodents (OECD, Paris, 2008).

All studies were carried out at Federal University of Mato Grosso (UFMT) Biochemistry laboratory belonging to Department of Chemistry (Cuiabá-MT, Brazil). The experimental procedures were conducted in compliance with the Brazilian College of Animal Experimentation (COBEA) and with the principles of Good Laboratory Practice as set forth in the OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring, Number 1, 'OECD Principles on Good Laboratory Practice' ENV/MC/CHEM (98) 17 (as revised in 1997). The study was approved by the UFMT Ethics Committee for Animal Use under number 23108101038/2015-91.

2.2.1 Animals

Female and male albino mice *Mus musculus* derived from the Swiss–Webster strain (20 – 25 g) and male albino rats *Rattus norvergicus*, Wistar strains (180 – 220 g) were used in these studies. The animals were provided by the Central Animal House of the UFMT. Prior to treatments, they were acclimated for 6 days in polypropylene cages. Throughout the trials, the animals were kept in the Animal Care Unity of the Biochemistry laboratory under controlled temperature (20 ± 1°C), relative humidity (50 ± 5%), 12 h light/dark cycle, and provided with water and feed (Nuvilab® autoclavable CR1 Sogorb, São Paulo, Brazil) *ad libitum* except when animals were fasted overnight prior to blood sampling or during behavioral testing.

2.2.2 Acute toxicity (Hippocratic test)

Acute oral assay of the encapsulated and non-encapsulated GCFE was performed in male (n=36) and female (n=36) mice in order to determine the lethal doses (LD_{50}), that is, the dose which kills 50% of a group of test animals, and also to determine tolerable dosage to conduct the subacute toxicity test in rats. Groups of 6 mice/sex/dose received singles dose of 100, 500, 1000, 2500, 5000 mg/kg bw or vehicle (distilled water, 1 mL/kg bw) by orogastric gavage, after an overnight fast of 12 h. The animals were monitored individually for clinical signs of toxicity or mortality by using the Hippocratic screen according to Malone & Robichaud (1962) in the open 5, 10, 15, 30, 60, 120, and 240 min following administration of powders, and daily for a period of 14 days. Body weight, food and water intake were measured daily. On the 15th day,

animals were euthanized by carbon dioxide asphyxiation and subjected to gross necropsy (i.e., examination of external surface of the body, all orifices, and the thoracic and abdominal cavities and their contents). Organs were removed to determine the relative weight [(organ weight / body weight) x 100].

2.2.3 Subacute toxicity

Male Wistar rats (n=40) were placed in individual metabolic cages and randomly distributed into one of the following groups (n=10/group): control group (C), rats treated with 100 mg/kg bw of encapsulated GCFE (En-$GCFE_{100}$), rats treated with 500 mg/kg bw of encapsulated GCFE (En-$GCFE_{500}$), and rats treated with 1000 mg/kg bw of GCFE (En-$GCFE_{1000}$). The control group received vehicle (distilled water, 1mL/100g bw) and the dose of encapsulated GCFE was adjusted with non-encapsulated GCFE using the fc 3.5. To dissolve microparticles in the vehicle, the powder was weighed daily into a conical tube and was solubilized in distilled water using a vortex mixer. The water volume added to solubilize the powder was calculated to achieve 1 mL solution/100 g body weight. Treatment was administered orally once a day for 30 days. Body weight of the animals, food intake and feces mass were measured once a week, and water consumption was determined every 3 days. The rats were also observed every two days for behavior alteration or for presence of any signs and symptom of toxicity. Near the end of the study, the rats were evaluated by functional observational battery (FOB) that assessed excitability, autonomic function, gait, and sensorimotor coordination, reactivity, sensitivity, and other abnormal clinical signs in an open field. At the end of the study, after fasting for 12h, the animals were euthanized, and the blood samples were collected in ethylenediaminetetraacetic acid (EDTA) Vacutainer® tubes for determination of complete blood count (CBC) using a veterinary hematology analyzer, Poch-100iV Diff (Sysmex of Brazil Ind. and Com. Ltd, Brazil), and in anticoagulant-free Vacutainer® tuber for determination of biochemical parameters (glucose (GLU), urea (Ur), uric acid (UA), creatinine (Cre), alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), amylase, total bilirubin (TBILI), direct bilirubin (DBILI), indirect bilirubin (IBILI), total cholesterol (CHO), HDL cholesterol, LDL cholesterol, triglycerides, alkaline phosphatase (ALP), total protein (TP), albumin and globulin) in an automatic biochemical analyzer Labmax Plenno (Labtest, Brazil) and hormonal cortisol as stress biomarker by chemiluminescence using a immunoassay system (Immulite® 1000, Siemens healthineers Brazil).

The liver, kidney, stomach, spleen, heart, lungs, and brain were removed and their relative weight [(organ weight/body weight)100] were determined before fragments being placed in 10% formalin for histological analysis.

2.2.4 Histopathological study

Histological sections of the liver, brain, testicles, stomach, lungs, spleen, heart, kidneys, and small and large intestine were processed by a conventional histopathological method. This was followed by staining with hematoxylin and eosin (H&E) and visualization by optical microscopy with a 40X objective. The animal tissues were examined blindly, and the criteria for assessing the occurrence of lesions in organs were done according to the methodology proposed Giordani et al (2015). Briefly, the criteria for liver injury were vacuolization of hepatocytes and pyknotic hepatocyte nuclei, number of Kupffer cells and enlargement of sinusoids. Moreover, the histopathological change of lungs was based on congestion, edema, inflammation and hemorrhage. The microscopic features of the organs En-GCFE-fed rats were compared with the control group.

2.2.5 Dose conversion between animals and human

The equation 1 described by Nair & Jacob (2016) was used to estimate human equivalent dose (HED) from the empirical approach and by using the NOAEL of encapsulated GCFE estimated in male rats.

$$\text{HED (mg/kg)} = \text{Animal dose (mg/kg bw)} \times K_m \text{ ratio}$$

Equation 1.

Where, animal dose is the NOAEL estimated by subacute toxicity test, and K_m ratio is obtained by dividing K_m human factor by animal K_m factor. The K_m factors are estimated by dividing the average body weight (kg) of human or rats by its body surface area (m^2). The average human body weight was considered 60 kg, and the body surface area 1.62 m^2. While the average animal body weight was the terminal body weight obtained at the end of the toxicological study, around 0.3 kg, and the body surface 0.043 m^2 (Nair & Jacob, 2016).

2.3 Behavioral test

Male mice (n=60) were treated with non-encapsulated GCFE and tested for locomotor activity by open-field test as described by Zomkowski et al. (2010). The experimental conditions include four doses of non-encapsulated GCFE (100, 500, 1000 or 2000 mg/kg bw, n = 6 per group), and control group (distilled water 1 mL/kg bw, n = 6 per group). The animals were pretreated with extract or vehicle twice, at 30 min and 1 h prior to the open-field test, and at the end of the test, the animals were euthanized by carbon dioxide asphyxiation.

The apparatus consisted of a wooden box (40×60×50cm) with visible lines dividing the floor into twelve equal squares drawn using a black sticker. The number of squares crossed with all paws (crossings) or rearing was counted in a 6-min session. The light was maintained at minimum and any interference such as noise was avoided during the test. Mice were individually placed into the center of the apparatus and allowed to explore freely while a blinded observer scored the number of times a mouse crossed one of the grid lines with all four paws or rearing occurrence (event).

2.4 Data analysis

GraphPad Software 5.00®, Inc. (GraphPad Software, Inc., San Diego, CA). was used for statistical analysis and plotting. The results of parametric tests were expressed as mean ± standard error of the mean (SEM), in experiments comparing more than two means, one-way analysis of variance (ANOVA) and the significance by F-*test* at 5% and 1% probability, as well as the contrast between means by Tukey-Kramer test were used to identify differences between treatments.

3. Results

3.1 Acute toxicity

3.1.1 Hippocratic screening

The Hippocratic screening provides a general estimate of pharmacological and toxicological nature of a determined compound (Lucio et al., 2000). For animal welfare reasons, the starting doses from 100 to 1000 mg/kg body weight of Non-En-GCFE and En-GCFE were used. No behavioral alterations or death were observed among animals in these dosages both in female and male mice. Mild analgesic effect was noted in doses from 500 to 1000 mg/kg bw both in Non-En-GCFE and En-GCFE treatment groups, but this effect was reversed around 4 h after a single dose administration both in male and female mice.

In agreement with OECD guideline 423 (2002), when available information suggests that mortality is unlikely at the highest starting dose level, then a limit test should be conducted. In this way, the dosages of 2500 mg/kg bw and 5000 mg/kg bw were assessed in a second step. As the additional upper dose level of 5000 mg/kg bw may be considered to identify substances which are relatively low acute toxicity hazard, under certain circumstances, it may present a danger for vulnerable population (OECD, 2008).

Non-En-GCFE at a dosage of 5000 mg/kg bw caused tonic convulsion followed by death of four male mice and three female mice. Two male mice died right after the administration of the maximum dose, and two more died around 4 hours after a period of ataxia. Among females, two showed an acute tonic convulsion episode and death afterward. One remained ataxic for around 8 hours and then died. However, doses of 2500 mg/kg bw of Non-En-GCFE and 5000 mg/kg bw of En-GCFE did not cause any death. But further effects on the autonomic system were noted, evidenced as Straub tail phenomenon accompanied by piloerection that was observed at the initial time (~30min) among both female and male mice. Besides, an initial excitation with an increased motor activity and respiratory rate were observed in the first 15 min among animals which received Non-En-GCFE and En-GCFE at 2500 mg/kg bw and 5000 mg/kg bw, respectively. Then, an increased fugue reaction and aggressiveness were observed (specially at 2500 mg/kg bw Non-En-GCFE and Enc-GCFE) in mice of both sexes. These side effects are characteristic of caffeine intoxication present with hyperventilation, dizziness, anxiety, tinnitus, tremor, and agitation (Rudolph & Knudsen, 2010).

Enophthalmos was seen after 60 min, along with palpebral ptosis (more sedation than a true blepharospasm) among great part of the animals treated with 5000 mg/kg bw of En-GCFE, and with 2500 mg/kg bw to 5000 mg/kg bw of Non-En-GCFE (the survivors). These effects were reversed after 8 hours of administration of encapsulated and non-encapsulated GCFE. No other deaths occurred after this period among the other treatment groups for 14-day observation.

3.1.2 Lethal Dose, 50% (LD_{50})

In accordance with the criteria established by the OECD guideline 423 (2002), the Non-En-GEC belong to hazard category 5 since LD_{50} was achieved in the dose of 5000 mg/kg bw, both to male and female mice. In this category are allocated substances which are of relatively low acute toxicity hazard having an oral or dermal LD_{50} in the range of 2000-5000 mg/kg or equivalent doses for other routes

The oral LD_{50} of chlorogenic acid and caffeic acid were tested by Schafer et al (1983) in red wing blackbird (sex and strain not provided), they reported the dosages of >100 mg/kg bw (0.282 mmol/kg) to chlorogenic acid and >100 mg/kg bw (0.555 mmol/kg) to caffeic acid. There is no information in the scientific literature which calculates a mean LD_{50} and standard deviation for these substances administered orally to rats, using studies performed under good laboratory practice (GLP) or equivalent. In a Non-Commercial Scientific and Production Partnership (NSPP, 2006), the acute oral toxicity of chlorogenic acid is reported to be > 2000 mg/kg bw in rats and >1580 mg/kg bw in mice. In a systematic review, Adamson (2016) reported that most accurate estimate of the acute LD_{50} of caffeine administered orally in male albino rats is 367 mg/kg bw. These data corroborate with the results found by *in silico* study on most of the compounds found in GCFE where caffeine was reported to have a lower LD_{50} when compared with other compounds, including 5-CQA (Faria et al., 2018c). In view of these findings, the content of caffeine found in Non-En-GCFE can be responsible to cause death in more than 50% of mice given that the dose of 5000 mg/kg bw provides around 400 mg/kg bw of caffeine. The fatal blood concentration of caffeine in human is 80 – 100 mg/L. Thus, in order to achieve this concentration, a person would need to ingest 5000 – 10000 mg of pure caffeine (Bonsignore et al., 2014). In addition, it has been speculated that caffeine's mechanism of death is usually associated with ventricular arrhythmia given that the molecular structure of caffeine is similar to adenosine and it can block the adenosine receptor. It leads to increases in the intracellular calcium concentration, causing noradrenaline release and sensitizing dopamine receptors, which can trigger arrhythmia (Yamamoto et al., 2015). Although the dosages of the En-GCFE are equivalent to Non-En-GCFE, it was not possible to determine the LD_{50} of encapsulated GCFE, probably due to the release delay of caffeine and other compounds from GCFE.

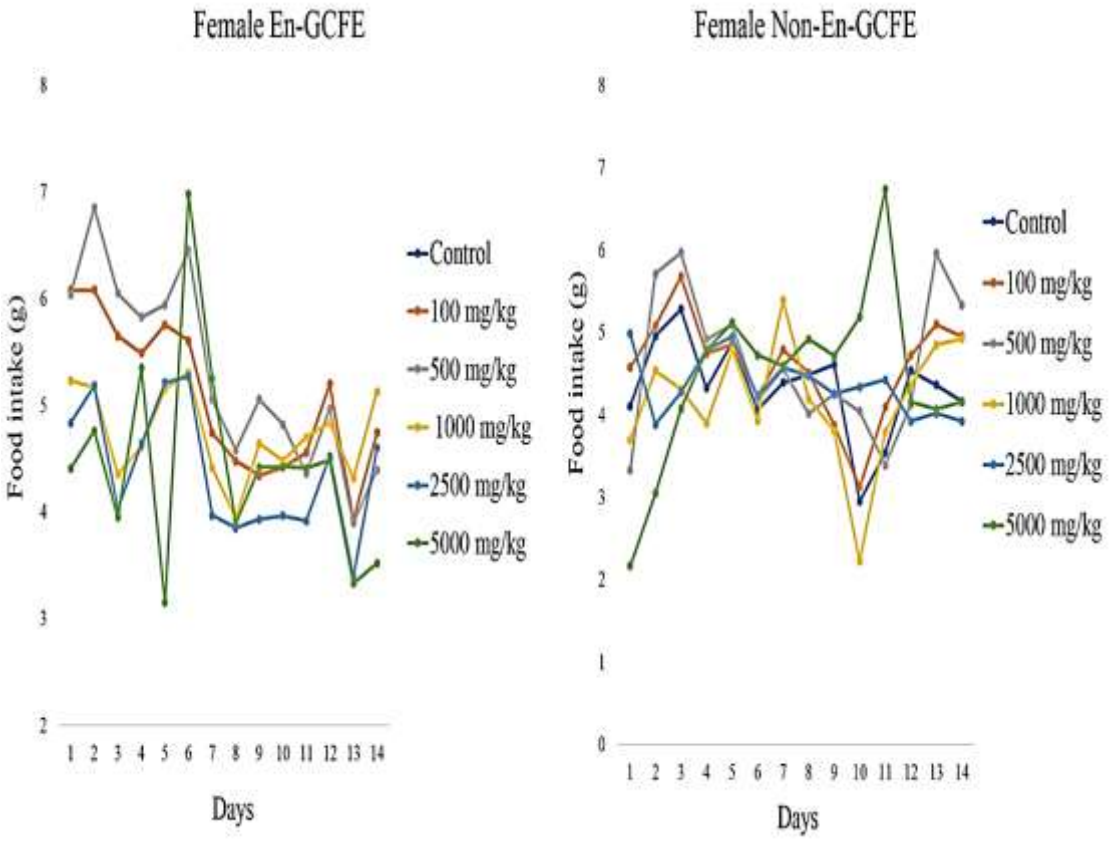
3.1.3 Body weight, food intake and water consumption

As shown in table 2, in the male group treated with 2500 mg/kg bw of En-GCFE the mean weekly body weight gains were significantly lower than those controls on days 7 and 14 ($p<0.05$). This effect was not dose-dependent since no difference was observed between the higher dosage with the control group. On the other hand, the female body weight was similar to the control group values all time-points.

The food intake (Figure1) and food efficiency (data not shown) were also similar among females in both treatments. But among males, the food efficiency was 31% lower at dosage of

2500mg/kg of Non-En-GCFE compared to the same dose of En-GCFE. On the other hand, among the males treated with Non-En-GCFE, the food intake and food efficiency were similar to the control group. The food efficiency is the ratio of body weight gained per gram of food eaten, and, it is well-known that there are sex-specific responses to the effects of voluntarily increased food intake by animals. In general, female animals present a higher percent increment of body fat than males (Jen et al., 1981). This information corroborates with findings of this study since females showed a higher food efficiency compared to male in all different dosages.

In relation to water consumption (Figure 2), despite the value variations in all point-time, in both sex and different treatment, the groups did not show a significant difference between them. Based on these results, the single doses of encapsulated and non-encapsulated GCFE did not interfere with food intake or water consumption.



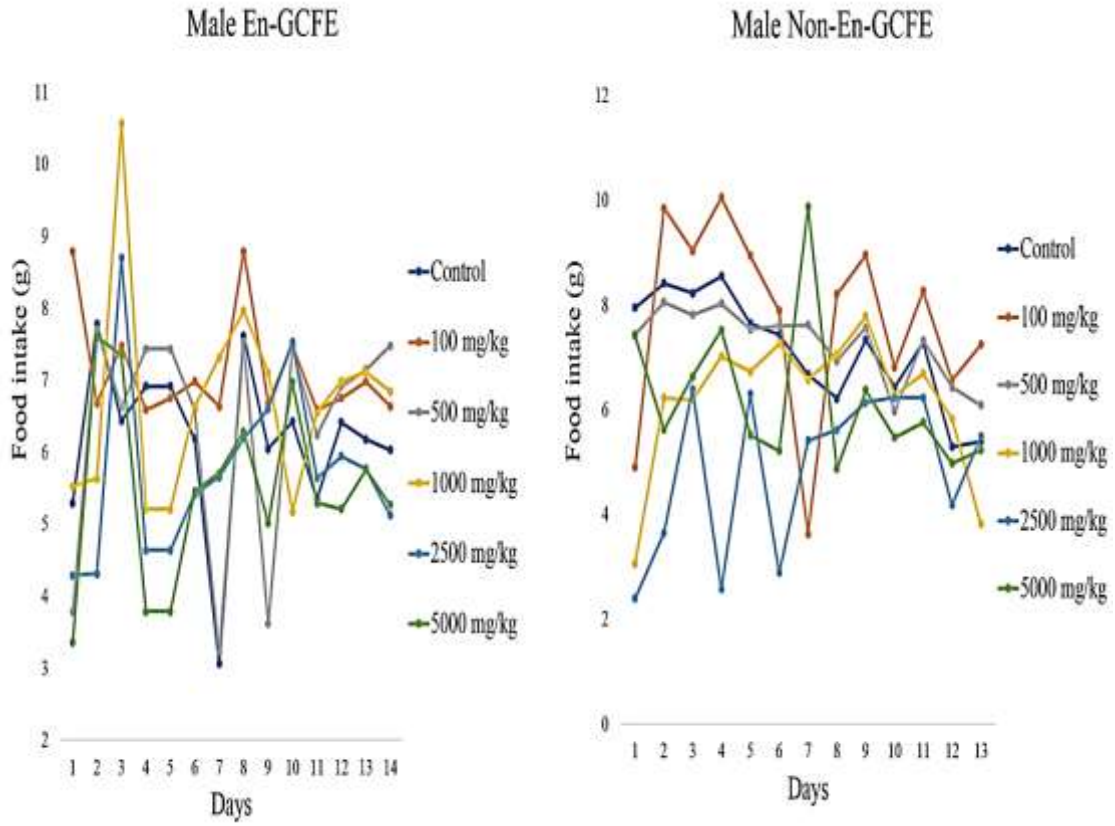


Fig 1. Mean food intake for 14 days from acute toxicity study after administration of single doses of encapsulated and non-encapsulated GCFE by female and male mice.

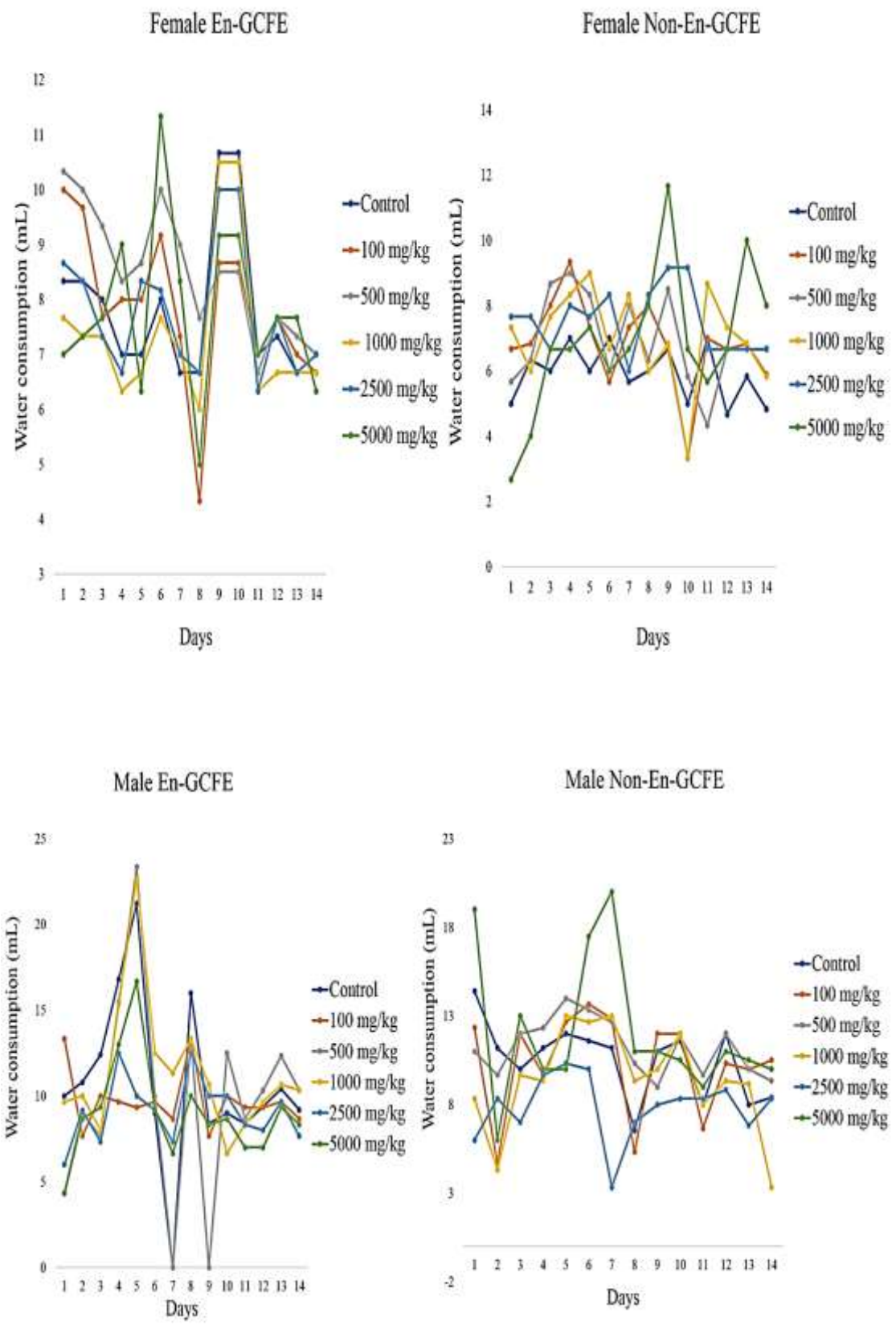


Fig 2. Mean water consumption for 14 days from acute toxicity study after administration of single doses of encapsulated and non-encapsulated GCFE by female and male mice.

Table 2. Toxicity results from acute oral administration of encapsulated and non-encapsulated extract from green *Coffea canephora* whole coffee fruit (0, 100, 500, 1000, 2500, 5000 mg/kg bw) in female and male rats for 14 days

Group	Dose (mg/kg bw)	Sex	Body weights (g)[a]			Clinical Signs	Macroscopical lesions findings	Mortality (%) (Dead/total)
			Day 0	Day 7	Day 14			
Non-En-GCFE								
		M	23.1±0.7	32.0±1.1	35.1±0.6	N	0	0% (0/6)
G1	**0**	**F**	25.4±0.8	29.8±0.7	30.6±0.88	N	0	0% (0/6)
		M	22.1±0.9	23.0±0.8	37.5±0.8	N	0	0% (0/6)
G2	**100**	**F**	22.7±1.2	26.7±1.0	28.5±1.18	N	0	0% (0/6)
		M	22.4±0.6	31.0±0.4	34.8±0.8	N	0	0% (0/6)
G3	**500**	**F**	24.6±0.9	29.0±0.8	30.2±0.9	N	0	0% (0/6)
		M	22.1±0.8	27.9±1.5	32.4±1.5	N	0	0% (0/6)
G4	**1000**	**F**	24.2±0.8	27.3±1.0	29.2±0.7	N	0	0% (0/6)
		M	22.7±0.6	33.3±1.07	37.0±1.3	Ab	0	0% (0/6)
G5	**2500**	**F**	24.3±0.6	26.2±1.0	28.5±0.7	Ab	0	0% (0/6)
		M	22.6±0.8	27.7±1.3	32.4±1.7	Ab	0	66.6% (4/6)
G6	**5000**	**F**	26.0±0.5	28.8±0.7	29.7±0.6	Ab	0	50% (3/6)

En-GCFE

		M	23.2±0.5	30.7±1.1	34.2±1.0	N	0	0% (0/6)
G1	**0**	**F**	23.2±0.6	28.0±0.9	30.0±0.8	N	0	0% (0/6)
		M	24.5±0.7	33.2±0.2	34.0±0.3	N	0	0% (0/6)
G2	**100**	**F**	22.0±0.5	28.0±0.5	31.1±0.4	N	0	0% (0/6)
		M	22.8±0.9	30.7±1.1	34.8±0.9	N	0	0% (0/6)
G3	**500**	**F**	23.7±0.7	28.5±0.8	30.6±0.6	N	0	0% (0/6)
		M	22.9±0.5	28.8±0.7	34.1±1.1	N	0	0% (0/6)
G4	**1000**	**F**	23.1±0.7	26.9±0.9	30.2±1.2	N	0	0% (0/6)
		M	23.7±0.3	24.6±0.3**	30.7±0.3**	N	0	0% (0/6)
G5	**2500**	**F**	24.9±0.4	26.1±0.6	28.4±0.5	N	0	0% (0/6)
		M	22.4±0.6	27.0±1.2	32.0±0.8	Ab	0	0% (0/6)
G6	**5000**	**F**	24.2±0.6	27.6±0.3	29.3±0.3	Ab	0	0% (0/6)

[a]The results were expressed as mean ± standard error of mean (SEM) of 6 rats. Analysis by ANOVA followed by Tukey-Kramer test. *p≤0.05 and **p≤001 represent significant differences from the control male or female groups. G1 = Control; G2 = Group 2 (100 mg/kg bw); G3 = Group 3 (500 mg/kg bw); G4 = Group 4 (1000 mg/kg bw); G5 = Group 5 (2500 mg/kg bw); G6 = Group 6 (5000 mg/kg bw); M = male; F = female; N = Normal; Ab = Abnormal. Non-En-GCFE = non-encapsulated GCFE; En-GCFE = encapsulated GCFE.

3.1.4 Relative organs weight

Changes in body and organ weights are a clear indicative of damage caused by the ingestion of a toxic substance. According to Piao et al. (2013) organ weight can be the most sensitive indicator of an effect of substance toxicity. As it can be seen in table 3, no significant differences in relative organ weight were observed between female groups treated with non-encapsulated and encapsulated GCFE ($p>0.05$), except to relative organ weight of liver in females treated with encapsulated GCFE at dosage of 1000 mg/kg bw, which showed a higher value compared to other groups. However, this effect was not dosage-dependent since the groups treated with 2500 and 5000 mg/kg bw do not show this alteration. In agreement with Uemitsu (1986), relative liver weight is a more sensitive toxicity indicator than absolute liver weight. There are several situations that lead to the increase of relative liver weight. Basically, the factors influencing liver weight are divided into two categories: those which cause enlargement without histopathological effects and those which cause enlargement as a sequel to liver injury by the presence of tumor, for example (Yeakel, 1948; Wilson et al., 2012). Enlargement of the liver without pathological alteration can be brought about by a variety of substances, many of which are metabolized by the liver such as drugs, food additives, insecticides, carcinogens, and normal body constituents as cortisone, thyroxine, estradiol, testosterone, and androsterone (Wilson et al., 2012). But in this oral acute toxicity, the necropsy of the liver compared to the control group did not show any macroscopic alteration, both in female and male groups.

In the male groups, significant differences ($p<0.05$) were observed to relative organ weight of kidneys at dosages of 500 and 5000 mg/kg bw of Non-En-GCFE, but not to 100, 1000 and 2500 mg/kg bw when compared to the control group. The groups treated with 500 and 5000 mg/kg bw of Non-En-GCFE showed a decrease in relative weight of the kidney compared to the control group. Although this effect does not appear to be dose-dependent, this should be observed in a longer study as subacute test. The decrease in organ weight mass can happen due to malnutrition, or even due to the decrease in water consumption, which can lead to kidney atrophy. However, in the present study, no difference was observed in neither food intake nor in water consumption among male or female groups. Gross examination in the main organs including the liver, spleen, stomach, kidneys, lungs, heart, and brain did not show any abnormal findings (data not shown). Therefore, these results indicate that the oral administration of a single dose until 2500 mg/kg bw did not show toxic effects in mice.

However, there were mild reversible reactions which may be suggestive of the action of caffeine on the central nervous system.

Even among the dead mice, no macroscopic alteration in selected organs nor relative organ weight alteration were observed. Despite being clear the role of caffeine in these fatal intoxications, specific organ alterations, i. e. brain edema and mild erosion with hemorrhage in the stomach, caused by caffeine overdose previously reported by Yamamoto et al. (2015) were not found in this toxicity study.

Acute toxicity generally provides initial information on the mode of toxic action of a substance, it also helps achieve a safe dose of a new compound and helps in dose determination in animal studies (Giordani et al 2015). For this study, the doses until 1000 mg/kg bw of encapsulated GCFE, that were better tolerable in the acute test, were selected to conduct a subacute study in male Wistar rats in order to help calculate the No Observed Adverse Effect Level (NOAEL) dose and to help for clinical studies.

Table 3. Relative body weights (%)[a] from the oral acute toxicity study of encapsulated and non-encapsulated GCFE in females and male mice

	Males (dose, mg/kg bw)						Females (dose, mg/kg bw)					
Organ/Groups	**Control**	**100**	**500**	**1000**	**2500**	**5000**	**Control**	**100**	**500**	**1000**	**2500**	**5000**
Non-En-GCFE												
Spleen (%)	0.60±0.04	0.49±0.01	0.54±0.08	0.48±0.02	0.51±0.05	0.52±0.13	0.69±0.08	0.61±0.01	0.59±0.02	0.61±0.03	0.44±0.02	0.53±0.05
Heart (%)	0.62±0.03	0.62±0.03	0.58±0.05	0.58±0.04	0.46±0.01	0.57±0.10	0.53±0.03	0.52±0.01	0.55±0.02	0.54±0.05	0.51±0.04	0.66±0.06
Stomach (%)	0.97±0.04	1.09±0.07	1.05±0.05	0.96±0.03	1.01±0.03	0.90±0.05	1.03±0.04	0.99±0.03	1.11±0.07	1.05±0.04	1.02±0.12	1.19±0.06
Liver (%)	5.14±0.19	5.27±0.18	5.09±0.20	5.01±0.16	4.85±0.19	4.70±0.17	5.46±0.23	4.92±0.11	4.56±0.09	4.51±0.21	4.71±0.21	4.82±0.25
Lungs (%)	0.66±0.04	0.63±0.02	0.64±0.04	0.76±0.04	0.63±0.02	0.65±0.02	0.82±0.04	0.67±0.02	0.75±0.03	0.74±0.03	0.72±0.02	0.91±0.03
Kidneys (%)	1.48 ±0.01	1.45±0.06	1.26±0.03*	1.38±0.04	1.30±0.05	1.25±0.04*	1.19±0.02	1.18±0.02	1.14±0.03	1.16±0.06	0.99±0.24	1.21±0.05
En-GCFE												
Spleen (%)	0.41±0.04	0.51±0.06	0.42±0.01	0.46±0.03	0.51±0.03	0.46±0.03	0.49±0.05	0.53±0.06	0.52±0.02	0.55±0.03	0.57±0.03	0.45±0.01
Heart (%)	0.46±0.04	0.54±0.02	0.51±0.02	0.54±0.01	0.57±0.02*	0.53±0.01	0.49±0.03	0.45±0.02	0.49±0.04	0.44±0.03	0.47±0.01	0.47±0.02
Stomach (%)	0.83±0.05	0.90±0.05	0.87±0.08	0.98±0.02	1.03±0.06	0.95±0.03	1.02±0.05	0.95±0.05	1.09±0.07	1.01±0.07	1.03±0.05	1.04±0.08
Liver (%)	6.23±0.69	6.47±0.21	5.94±0.05	5.48±0.24	6.31 ± 0.68	5.09±0.12	5.14±0.14	4.98±0.12	5.19±0.15	5.45±0.14*	4.55±0.18	4.64±0.22
Lungs (%)	0.62±0.05	0.75±0.03	0.70±0.04	0.79±0.04	0.68±0.02	0.77±0.06	0.70±0.04	0.63±0.02	0.67±0.01	0.67±0.01	0.66±0.02	0.72±0.02
Kidneys (%)	1.33±0.01	1.39±0.06	1.36±0.05	1.37±0.01	1.49±0.03	1.32±0.05	1.19±0.03	1.10±0.04	0.90±0.18	1.16±0.03	1.10 ±0.01	0.96±0.12

[a]Relative body weight (%) = ((g/g body weight) x 100). The results were expressed as mean ± standard error of mean (SEM) of 6 mices. Analysis by ANOVA followed by Tukey-Kramer test. *$p \leq 0.05$ and **$p \leq 001$ represent significant differences from the control group.

3.2 Subacute toxicity

Before *in vivo* studies, the previous study conducted by Faria et al. (2018c) assessed the potential hazards for major compounds found in GCFE (i. e., 5CQA, caffeic acid, *p*-coumaric acid, caffeine, and trigonelline) by computer simulation. The *in silico* screening revealed no toxicological potential for most of the parameters assessed, including carcinogenicity, hepatotoxicity, and skin sensitization. However, 5-CQA was predicted to cause more adverse effect than caffeine, but these results are not clear since there is little information about the safety of chlorogenic acids in the literature. Besides, in agreement with Faria et al. (2018c), the toxicological profile observed for the GCFE may be influenced by a synergistic effect of the compounds. In this way, the *in vivo* data are essential to the assurance of the safety of GCFE' products.

Repeated dose toxicity testing is carried out for a minimum of 28 days, and the test substance is administered daily through the oral route, preferentially (OECD, Guideline 407). Usually, a rodent of any gender and age 5–6 weeks is used for repeated dose toxicity testing (Parasuraman, 2011). In this study, male rats were chosen to conduct the subacute test. Encapsulated GCFE were administered orally in via gastrogavage for 30 days. During this period, no treatment-related clinical symptoms or death were recorded. Autonomic and central nervous system, somatomotor activity and behavior pattern were found to be normal in all the experimental groups.

The subacute toxicity test was assessed only with encapsulated GCFE due to the fact that the encapsulated GCFE demonstrates to have less adverse effect when compared to non-encapsulated GCFE in Hippocratic screening.

3.2.1 Body weight, food intake and water consumption

There were no significant differences in the body weight, water consumption, weight of feces, food intake (figure 3), or feeding efficiency (data not shown) in treated animals compared to the control group ($p>0.05$). The encapsulated GCFE did not affect gastrointestinal transit since symptoms like diarrhea or intestinal constipation were not observed. The feces mass increased at the last time-point in accordance with the growth of the animals.

A minimal variability in food intake, body weight, and water consumption were seen throughout the study period (Fig 3A; 3B; 3C). A decrease (but not a significant one) in body weight gain was observed after 7 weeks and in the last week of the test, generally indicating

food avoidance, which would have happened due to some discomfort caused by the gastrogavage, but not as a result of the encapsulated GCFE consumption, but these episodes were observed among the animals of the control group. Differently, in the subchronic study carried out by Heimbach et al (2010) significant body weight gain, food consumption, and food efficiency were observed throughout the 90-day dietary study at dosages of 3446 and 4087 mg/kg bw/day. However, these were not considered adverse or toxicologically significant by authors.

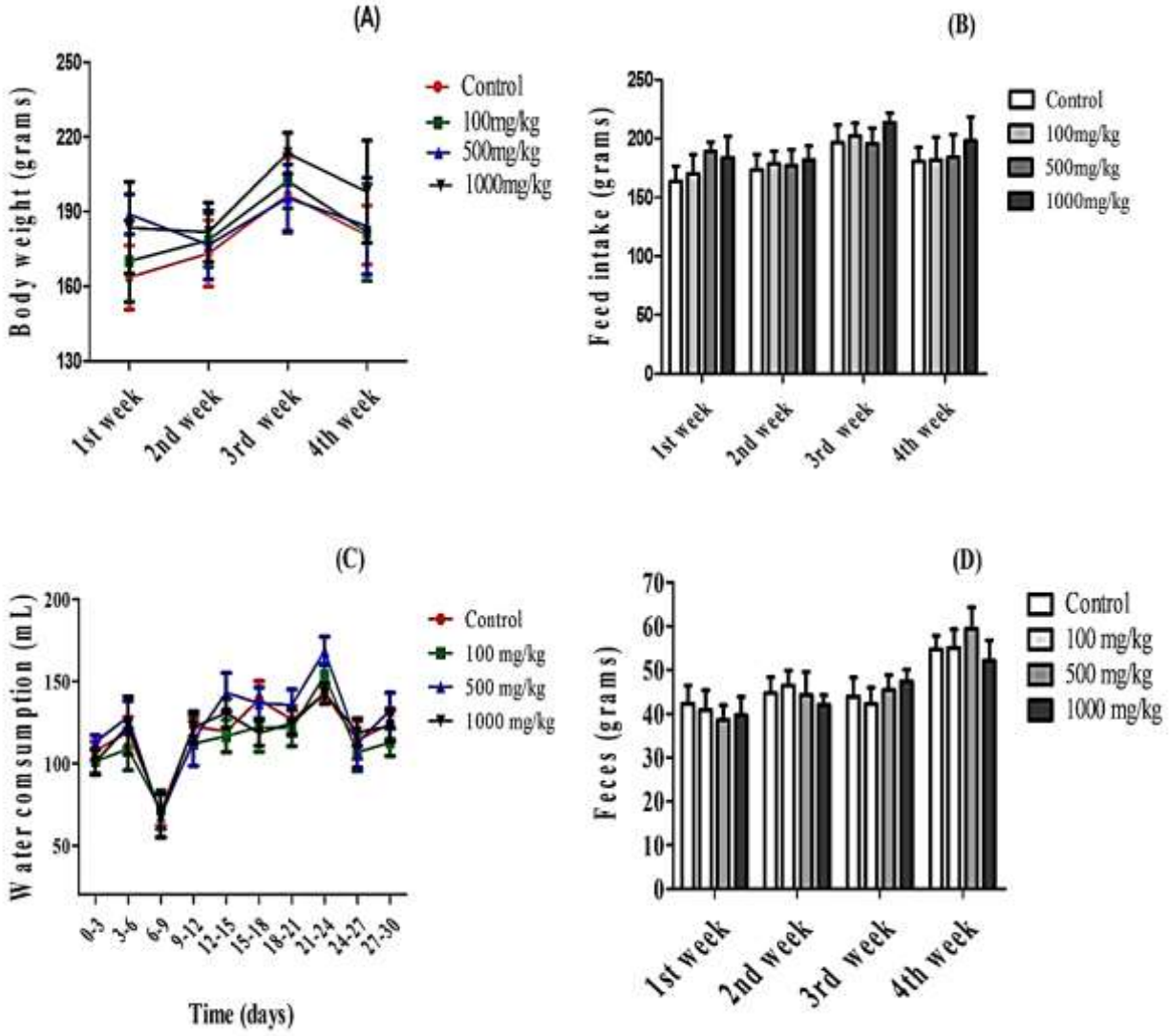


Fig 3. Results of body weight gain (A), feed intake (B), water consumption (C) and feces mass (D), from the 30-days subacute toxicity study with Wistar rats (males) treated with encapsulated GCFE.

3.2.2 Relative and absolute organs weight and histopathological findings

The terminal body weight of rats did not show significant difference ($p>0.05$) when comparing the treated groups with the control group (Table 4). In the subacute study, the decrease in body weight mass is a sign of systemic toxicity (Teo et al., 2002). Besides, the decrease in the ponderal development, the systemic toxicity manifests itself through the decrease in food and water consumption, behavioral alteration, apathy, bad condition of the coat such as hair loss, biochemical and hematological alterations, and relative organ weight alterations (Gonzales & Silva, 2003; Cunha et al., 2009). The encapsulated GCFE did not cause any significant alterations in body weight, food and water consumption as shown in figure 3 when compared to the control group ($p>0.05$).

No significant difference was found in relative or absolute organ weights as can be seen in table 4. These findings are not in agreement with the results achieved by Heimbach et al. (2010), which found some statistically significant changes in absolute and relative organ weights (e.g., kidneys, heart, and liver) in mid-dose (~2000 mg/kg bw/day) and high-dose (~4000 mg/kg bw/day) of ethanol extract from whole coffee fruit incorporated at animals dietary.

Figure 4 shows photomicrograph of some of main organs analysed histologically. The section of heart, liver and kidney tissue stained with H&E revealed normal morphology without inflammatory cell infiltration. In all groups, including control, some alterations were seen in the superficial tissue of the stomach compatible with the colonization of Candida sp., however, other stomach regions showed no alteration.

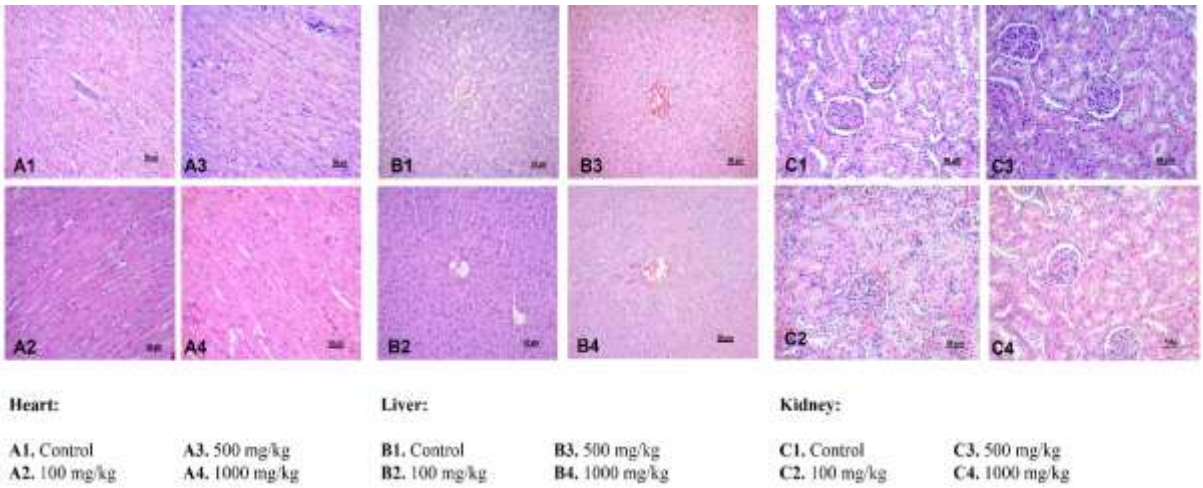


Fig 4. Photomicrograph of heart, liver and kidney (50 μ) analyzed histologically.

Table 4. Terminal body weight and absolute and relative main organs weights[a] from subacute toxicity assessment of the encapsulated GCFE with male Wistar rats.

	Treatment			
	Control	100 mg/kg	500 mg/kg	1000 mg/kg
Terminal body weight (g)	296.3±10.2	298.2±8.8	300.9±12.2	296.9±7.8
Brain weight (g)	1.91±0.04	1.84±0.05	1.86±0.05	1.84±0.04
Relative brain weight (%)[a]	0.65±0.02	0.62±0.02	0.62±0.03	0.62±0.02
Liver weight (g)	12.27±0.60	12.96±0.55	13.04±0.66	12.16±0.70
Relative liver weight (%)	4.12±0.08	4.34±0.13	4.33±0.14	4.07±0.12
Heart weight (g)	1.21±0.05	1.24±0.05	1.16±0.05	1.18±0.05
Relative heart weight (%)	0.40±0.01	0.41±0.02	0.38±0.01	0.39±0.01
Lungs weight (g)	2.16±0.16	2.37±0.12	2.14±0.08	2.08±0.13
Relative lungs weight (%)	0.72±0.04	0.80±0.04	0.72±0.03	0.70±0.05
Stomach weight (g)	1.97±0.07	1.88±0.08	1.93±0.08	1.88±0.12
Relative stomach weight (%)	0.67±0.02	0.63±0.03	0.65±0.04	0.63±0.04
Spleen weight (g)	0.81±0.04	0.81±0.04	0.80±0.04	0.80±0.03
Relative spleen weight (%)	0.27±0.01	0.27± 0.01	0.26±0.06	0.27±0.01
Kidney weight (g)	2.84±0.11	2.91±0.10	3.01±0.10	2.87±1.13
Relative kidney weight (%)	0.96±0.03	0.98±0.02	1.00±0.02	0.96±0.02
Testis weight (g)	3.75±0.17	3.37±0.01	3.36±0.15	3.31±0.15
Relative testis weight (%)	1.27±0.06	1.13±0.03	1.13±0.07	1.12±0.07

[a]Relative body weight (%) = ((g/g body weight) x 100). The results were expressed as mean ± standard error of mean (SEM) of the 6 rats. Analysis by ANOVA followed by Tukey-Kramer test. *$p \leq 0.05$ and **$p \leq 001$ represent significant differences from the control group.

Some studies reported about the effect of coffee and their compounds, such as caffeine and chlorogenic acid in gastrointestinal system (Cohen & Booth, 1975; Boekema et al., 1999; Welsh et al, 2015). Both coffee and caffeine can stimulate the hypersecretion of gastrin and gastric acid secretion causing mild erosive and hemorrhagic points at stomach (Boekema et al., 1999; Yamamoto et al., 2015). Meanwhile, decaffeinated coffee is found to be even more acidic than caffeinated coffee due to the fact that higher concentration of organic acids as chlorogenic acid provided by Robusta coffee, commonly used to produce decaffeinated coffee (Sinrém et al, 2001). Despite the high content of chlorogenic acid and caffeine in the GCFE, the encapsulated GCFE did not cause any macro or microscopical damage in the architecture of the stomach mucosa. Probably, this could be explained by the protection offered by the wall material, composed by the mixture Arabic gum (GA) and maltodextrin (MD), which could avoid substantially the contact of GCFE with the superficial tissue of the stomach. The role of

GA as prebiotic and antioxidant agent has been studied widely (Phillips & Phillips, 2011; Rodrigues et al., 2012; Kaddam et al., 2017). Besides, the combination of MD to GA has been proposed as phenolic protector agent (Khazaei et al., 2014; Ballesteros et al., 2017; Faria et al., 2018c). According to McRorie & McKeown (2017), the GA is advantageous for targeted delivery because it remains intact in the stomach and the small intestine.

In the present study, no alteration in the architecture or inflammatory infiltration was observed in the large or small intestine structures. On the other hand, Du et al. (2013) reported that high dose of chlorogenic acid (7 mg/kg bw) administered via parenteral caused severe architecture damage manifested by loss of the ileum villi, villus congestion, and massive infiltration of inflammatory cells. According to the authors, these lesions were triggered by oxidative stress observed by ROS increasing and, consequently, enhancement of inflammatory mediators in intestinal endothelium. High-dose of ethanol extract from whole coffee fruit, up to 4000 mg/kg bw (~40% phenolic acids and 0.6 – 9.0 % caffeine) administered orally for 14 days, caused colon/intestinal distention 3 of 10 female rats, but it was not considered a toxic effect by the author given that no histopathological alteration was seen (Heimbach et al., 2010).

The high-dose (1000 mg/kg bw/day) of encapsulated GCFE provides around 66 mg/kg bw of chlorogenic acids. However, the patterns of lesion found by Du et al. (2013) caused by chlorogenic acid were not observed in the present study between the treated or the control animals, maybe because the administration via was different among studies, and due to the microencapsulation process, that could protect the intestine endothelium of chlorogenic acids and caffeine present in GCFE.

In a chronic study conducted by Chan et al. (2010), where the administered by gavage green tea extract with standardized content of caffeine (4.99%, ~15 mg/kg bw in 1000 mg/kg of extract), liver alterations were found in 3 of 10 female rats that received 1000 mg/kg for 3 months. In agreement with the authors, the caffeine component of the tea extracts was responsible for this side effect, considering that caffeine alter the CYP450 and is a potent inducer of CYP1A2 (Goasduff et al., 1996). In the present study, the encapsulated GCFE provides around 24 mg/kg bw. However, for 30 days of treatment, no macroscopic or histological liver alteration were observed among treated or control male rats.

Encapsulated GCFE did not cause structural alteration in testis, corroborating with findings reported by Heimbach et al. (2010). Despite no alteration in weight of organs, some alteration findings in brain and lungs were seen in both the treated and the control animals. The brain section at diencephalon and midbrain junctions, and in rostrocaudal region showed mild

neutrophil vacuolization and mild acute neural necrosis. These lesions are presumably caused by euthanasia protocol adopted in this study since CO_2 euthanasia increased brain glutamate levels postmortem (Gos et al., 2001). In addition, anesthetic levels of CO_2 caused hemorrhage and acidosis in the brain (Karmarkar et al., 2010). Moderate alveolar wall thickening with the presence of mononuclear and polymorphonuclear cell infiltrates without the presence of bacterial agent in lungs tissue were also found among all animal groups, including the control one, but these findings are commonly observed in enclosed individuals (Department of Primary Industries of Australia, 2004). No histological alterations in treated groups were significant compared to the control animals, these findings exclude the role of encapsulated GCFE in any toxic tissular effect.

3.2.3 Biochemical and hematological parameters

The consumption of encapsulated GCFE in did not cause any alteration in hematological parameters among the treated groups and these compared to the control animals. Heimbach et al. (2010) found only mild significant alterations in mean platelets concentration when used mid (2030 mg/kg bw) and high (4087 mg/kg bw) doses of the extract from whole coffee fruit after 90-day dietary study. Small changes in biochemical parameters were observed among the treated groups compared to the control one. Mean albumin concentration was found decreased in the highest dose of encapsulated GCFE compared to the control group ($p<0.05$). Besides, statistically significant and dose-dependent increase in glucose concentration was seen among the groups treated with 500 and 1000 mg/kg bw of encapsulated GCFE compared to control ($p<0.05$). Increased glucose level was also found by Heimbach et al. (2010), however, only in low dose (965 mg/kg bw) of coffee fruit extract.

All groups showed a serum albumin below the reference range proposed by Giknis & Cilfford (2008). Albumin is a body's predominant serum-binding protein which transports various substances, including bilirubin, fatty acids, metals, ions, hormones, and exogenous drugs (Lehninger et al., 2013) and the hypoalbuminemia can be caused by various conditions including malnutrition, nephrotic syndrome, hepatic cirrhosis, heart failure, and more commonly caused by acute and chronic inflammatory responses (Gatta et al., 2012). However, as it can be seen in table 6, no other hematological or biochemical alteration corresponding with possible causes of hypoalbuminemia was found between animals since blood cells that indicate inflammatory process, or biomarkers that measure the liver and also heart function such as enzymes aspartate aminotransferase (AST), alanine aminotransferase, and alkaline phosphatase (ALP), or more specific marker of liver function as (ALT), γ-glutamyl transpeptidase (GGT)

bilirubin total (TBILI), bilirubin direct (DBILI) and indirect (IBILI), and renal function as urea (Ur), creatinine (Cre), uric acid (UA) are not increased compared to the control group. Besides, lab markers of malnutrition as globulin and total protein decreased, diminishing total cholesterol levels, and diminished the lymphocytes count (Bharadwaj et al., 2016). Such alterations were not significant when compared to the control group ($p>0.05$).

The dose-dependent enhancement of serum glucose does not corroborate with *in vitro* antidiabetic effect of green coffee extract reported by Henry-Vitrac et al. (2010). However, a number of studies show the role of coffee compounds as trigonelline and chlorogenic acids and regular consumption of coffee with a lower risk of type 2 diabetes mellitus in humans (van Dam, Feskens, 2002; McCart, 2005; Huxley, 2009; van Dijk et al, 2009) and with a antihyperglycemic effect in diabetic rats (Karthikesan et al., 2010). In contrast, the findings of the present study do not demonstrate this profile in heathy rats.

There are documented data about action of caffeine and coffee to elevate the stress hormones cortisol, epinephrine, and norepinephrine (Lane et al., 1990; Krieger et al., 2016). However, the 30-day oral administration of this encapsulated GCFE did not show a statistically significant increase in cortisol level compared to the control group ($p>0.05$).

In summary, these hematological and biochemistry results suggesting that oral administration of encapsulated GCFE did not produce any adverse effect in these parameters corroborate with findings in histopathological analyses, which showed a normal tissue architecture in all selected organs. Besides, no alteration clinical signs and behavior were observed among the treated and control groups, even in the highest dose. These findings indicate that the NOAEL of encapsulated GCFE is 1000 mg/kg bw/day for male rats.

Considering the human equivalent dose, obtained by equation 1, the safe dose established to humans is 189 mg/ kg bw/ day or 11.34 grams considering an adult with 60 kg of body weight mass. It is worth mentioning that this equation assumes body surface area as a unique important characteristic, and exclude physiological, and biochemical process among species, besides excluding the possible difference in pharmacokinetics/physiological time.

Table 6. Effects of the ingested of encapsulated GCFE by male rats after 30-day in haematological and biochemical parameters.

Hematological parameters	Control	100 mg/kg	500 mg/kg	1000 mg/kg	Reference[1]
Red blood cell (10^6/mL)	8.81±0.21	8.91±0.27	8.76±0.19	8.18±0.30	7.27-9.65
Hemoglobin (g/dL)	16.09±0.40	16.31±0.57	15.95±0.21	14.92±0.44	13.7-17.6
Hematocrit (%)	51.44±1.22	51.67±1.89	50.88±0.87	46.62±1.73	39.6-52.5
MCV (m^3)	58.51±1.27	58.01±1.34	58.21±1.21	57.08±1.11	48.9-57.9
MCH (pg)	18.28±0.34	18.33±0.44	18.25±0.37	18.30±0.37	17.1-20.4
MCHC (%)	31.28±0.28	31.60±0.35	31.37±0.24	32.07±0.32	32.9 – 37.5
Leukocytes (10^3/µl^3)	6.67±1.58	9.61±0.83	6.77±0.86	7.7±1.01	1.96 – 8.25
Segmented (%)	21.56±6.04	14.44±1.11	16.88±1.34	15.5±1.10	6.2-27.6
Eosinophils (%)	6.33±0.92	7.67±0.78	7.33±0.37	8.00±0.73	0.2-3.5
Basophils (%)	0	0	0	0	0
Lymphocytes (%)	70.11±6.63	76.33±1.43	70.55±1.27	75.25±1.08	66.6-90.3
Monocytes (%)	1.33±0.16	1.56±0.17	1.88±0.77	1.25±0.16	0.8-3.8
Platelets (10^3/µL)	748.89±76.12	814.22±60.48	871.22±58.09	792.04±79.04	638-1177
biochemical parameters					
Glucose (mg/dL)	72.32±7.65	77.77±4.60	88.31±9.50*	90.95±12.67*	70 -208
Ur (mg/dL)	51.54±3.46	53.22±3.49	45.28±2.11	43.28±3.04	10.7 - 20
Cre (mg/dL)	0.77±0.04	0.80±0.03	0.71±0.01	0.68±0.01	0.3-0.5
CHO (mg/dL)	100.11± 4.19	110.65±7.30	99.55± 6.10	97.01±7.98	37-95
HDL (mg/dL)	43.98±2.40	46.73±1.79	43.24±2.33	40.46±3.05	-
LDL (mg/dL)	46.32±2.68	49.58±7.40	43.20±6.01	50.16 ±7.79	-
VLDL (mg/dL)	9.80±0.75	11.96 ±1.60	10.56 ±1.41	10.80±1.11	-
TG (mg/dL)	53.10±2.88	66.44 ±6.93	65.55±8.43	54.30±3.92	27-140
UA (mg/dL)	1.03±0.08	1.41±0.23	1.50±0.36	1.17±0.22	-
TBILI (mg/dL)	0.10±0.02	0.09±0.03	0.10±0.03	0.13±0.05	0.05-0.15
DBILI (mg/dL)	0.03±0.00	0.02±0.00	0.02±0.00	0.02±0.00	0.03-0.05

IBILI (mg/dL)	0.07±0.01	0.07±0.02	0.07±0.03	0.10±0.05	0.01-0.12
TP (g/dL)	7.65±0.22	7.60±0.18	7.25±0.20	7.07±0.18	5.2-7.1
Alb (g/dL)	3.11±0.07	3.24±0.07	2.96±0.07	2.73±0.11*	3.4-4.8
Globulins (g/dL)	4.53±0.17	4.34±0.12	4.28±0.15	4.33±0.18	1.58-2.67
ALT (UI/L)	57.76±6.43	66.28 ±3.73	60.24±6.47	58.27±8.01	18-45
AST (UI/L)	130.20±26.58	137.84±23.80	125.36±23.13	129.38±26.13	74-143
AMI (UI/L)	468.48 ± 45.73	480.01±38.46	493.64±37.57	442.18±25.37	-
GGT (UI/L)	6.73±0.85	5.74±0.75	7.90±0.97	7.07±0.58	-
ALP (UI/L)	121.15±10.45	103.18±9.04	102.91±11.14	103.08±6.54	62-230
COR (mg/dL)	0.81±0.02	0.82±0.02	0.84±0.02	0.84±0.02	-

Results are expressed as mean ± standard error of mean (SEM) of the 10 analysis One-way ANOVA followed by Tukey-Kramer test. * $p \leq 0.05$ and ** $p \leq 0.01$ represent significant differences from the control group. Difference ($P \leq 0.05$) between groups of the same treatment represented by the letters [a], [b] and [c]. Glu, glucose; AMI, amylase; Ur, urea; LDL, low density lipoprotein; VLDL, very low-density lipoprotein; HDL, high density lipoprotein; TG, triglycerides; CHO, total cholesterol; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, γ-glutamyl transferase; Alb, albumin; TP, total protein; UA, uric acid; Cre, creatinine; ALP, alkaline phosphatase; TBILI, total bilirubin; DBILI, direct bilirubin; IBILI, indirect bilirubin, FAL, alkaline phosphatase. COR, cortisol. [1]Giknis & Cilfford (2008)

3.3 Behavioral test

Due to the autonomic effects observed among mice after an oral single dose of 2500 and 5000 mg/kg bw of non-encapsulated GCFE, the maximum limit dose selected to the open-field test was 2000 mg/kg bw.

In the study proposed by Nehlig et al. (1992), caffeine supplied in gavage exerted muscle relaxant activities and sedative effects or psychostimulant effects. However, these possible nonspecific effects on non-encapsulated GCFE evaluated in the present study, by the open field test, neither changed the number of crossings nor reared when compared to the control group after 60 min of administration until dose of 1000 mg/kg bw in rearing and 2000 mg/kg bw in the crossing parameter (Figure 5). But a statistically significant ($p<0.01$) increase in the number of crossing was observed after 30 min between animals that received 1000 mg/kg bw of non-encapsulated GCFE compared to control animals. Besides, animals treated with 1000 mg/kg bw showed a significant decrease in rearing number after 30 min ($p<0.05$) and the group treated with 2000 mg/kg bw decreased significantly the number of rearing after 30 and 60 min of administration of non-encapsulated extract (Figure 5).

In a previous study, Czok and Lang (1961) reported that chlorogenic acid alone had a weak neurostimulating effect in rats, but chlorogenic acid co-administered with caffeine showed an enhanced central stimulant action, dose-dependently. A more recent study by Ohnish et al (2006) assessed the effect of chlorogenic acid and their metabolites as caffeic acid and *m*-coumaric acids on spontaneous locomotor activity in mice. They concluded that chlorogenic acid has a weak caffeine-like psychostimulant property, but the dose of 2.8 mmol/kg of this compound showed a significant increase in locomotor activity. However, it was found that the metabolites caffeic acid and *m*-coumaric acids showed more potent effects in increasing locomotor activity than chlorogenic acid. In a clinical random trail, adult males who received 200 mg of caffeine from green coffee extract provoked an increase in epinephrine similar to the control, and did not show significant change in blood pressure or heart rate from baseline levels at 60 and 120 min post-GCFE dose, generating a hypothesis that natural caffeine sources may have different impacts on excitatory neurotransmitter, particularly epinephrine response (Krieger et al., 2016).

In the present study, the stimulant effect of GCFE rich in caffeine and chlorogenic acid was seen only by a number of crossings after 30 min of receiving a dose of 1000 mg/kg bw, but not in the number of rearing. On the other hand, the dose of 2000 mg/kg bw showed a significant neuro-depressive effect compared to other treated groups ($p<0.05$) (data not show), but not

compared to the control one related to the number of crossings ($p>0.05$). These results may be associated with some discomfort caused by the extract at this dosage such as seen at 2500 mg/kg dosage in the Hippocratic screening, given that a high dose of caffeine can cause hypotension, dizziness, nausea, among other symptoms (Yamamoto et al., 2015).

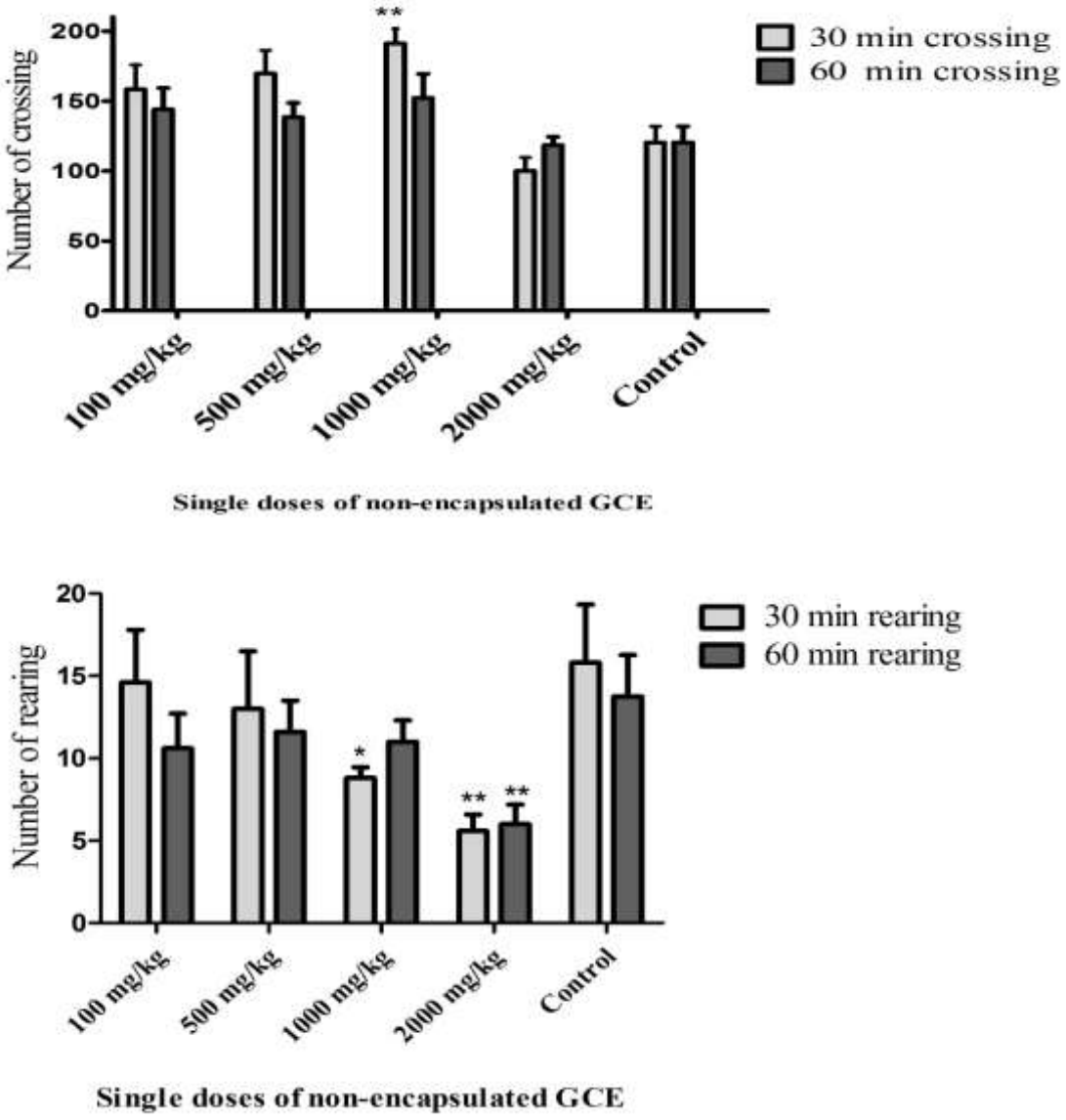


Fig 5. Number of crossing and rearing from open field test carried out after 30 and 60 min of singles doses of non-encapsulated GCFE in rats. Results are expressed as mean ± standard error (SEM) of the 6 samples. One-way ANOVA followed by Tukey-Kramer test. * $p \leq 0.05$ and ** $p \leq 0.01$ represent significant differences from the control group

4. Conclusion

The acute toxicity tests provided important information on the safety of encapsulated and non-encapsulated GCFE. The $LD_{50,}$ in mice, was determined to be 5000 mg/kg bw. Despite the

fact that the encapsulated form can be calculated to contain theoretically the same amount of chemical marker 5-CQA, it was not possible to determine the LD_{50} of encapsulated GCFE, presumably due to the delay in the release of the caffeine from GCFE. The doses of 100, 500 and 1000 mg/kg bw of encapsulated GCFE, calculated to provide the same amount of chemicals in non-encapsulated form, were selected to assess the subacute safety in male Wistar rats because in Hippocratic screening, these doses showed fewer side effects.

In the subacute toxicity assay, the oral administration for 30 days of the highest dose (1000 mg/kg b/day) of encapsulated GCFE did not produce any adverse effects. This result indicates that encapsulated GCFE could be safe for daily consumption, since the no-observed-adverse-effect level (NOAEL) in this study is considered to be 1000 mg/kg bw/day for male rats. And the HED for an adult of 60 Kg of body weight mass was calculated to be 189 mg/ kg bw/ day or 11.34 grams/day.

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CAPITULO VII

DISCUSSÃO GERAL

O café é uma das culturas agrícolas de maior importância no cenário agronômico nacional. O subproduto mais consumido desta planta são as sementes torradas e moídas para a produção da bebida do café. No entanto, o fruto inteiro do café, principalmente nos primeiros estágios de maturação, tem sido reconhecido por conter alto teor de fitonutrientes biologicamente importantes, incluindo compostos antioxidantes, anticancerígenos, antiobesogênico, entre outros. O fruto verde é caracterizado pela cor verde-cana e pela presença de grandes quantidades de compostos fenólicos que geram sabor adstringente e aroma indesejáveis à bebida do café. Por isso, a presença de frutos verdes juntamente aos frutos cereja no processamento dos grãos para a produção do pó para bebida é indesejável.

Grande parte dos bioativos do café são descartados com a fruta quando os grãos do café são processados, ou destruídos durante o processo de torra dos grãos do café. As espécies de café com maior valor de mercado são a *Coffea arabica* e a *Coffea canephora* conhecida também como Café Robusta, sendo esta última mais aplicada a *blends* e no processo de descafeinação, para produzir café descafeinado. O café Robusta apresenta maior conteúdo de ácidos clorogênicos e cafeína comparado ao café arábica, isso foi comprovado neste estudo, corroborando com dados da literatura, através da quantificação destes compostos por cromatografia liquida de alta eficiência (CLAE). O método de quantificação e identificação utilizado para analisar os componentes químicos do café já havia sido validado neste mesmo laboratório para analisar o perfil químico de grãos do café cru, no entanto, neste estudo o mesmo método foi validado para os extratos do fruto do café verde uma vez que as matrizes são diferentes. O fruto do café verde apresenta maior quantidade de compostos interferentes quando comparado ao grão visto que no uso do fruto não são descartadas a casca, a polpa, a película prateada e o pergaminho. No ensaio de validação a porcentagem de recuperação foi acima de 98% para o ácido 5-*O*-CQA, cafeína e trigonelina, com um desvio padrão menor de 5% para todos esses compostos. A robustez do método também foi avaliada em diferentes temperaturas do forno do cromatógrafo, variando ±1ºC, com uso de dois diferentes acidificantes na fase móvel e utilizando dois diferentes lotes de coluna cromatográfica. Ambos apresentaram desvios padrão de 2 a 7% em relação a área e ao tempo de retenção dos compostos, respectivamente.

Diante disso, este trabalho veio ao encontro de explorar a matriz do fruto verde da espécie Robusta, no estágio de maturação II (chumbão), e integro do café para a obtenção de extratos fluidos, secos e microencapsulados. Para isso, etapas dos processos de obtenção de cada produto supracitados tiveram de ser definidas neste estudo uma vez que não existem dados a respeito

do processo de obtenção do extrato fluido, seco ou microencapsulado, usando o fruto do café no estágio de maturação chumbão, na literatura.

Inicialmente, foram definidos os parâmetros de secagem e obtenção do pó do fruto do café verde. Diferentes temperaturas e tempos de secagem foram testados a fim de alcançar a umidade ideal para a moagem do fruto e obtenção de pó com propriedades granulométricas que oferecessem extensa superfície de contato, no entanto, não muito finas a ponto de causar entupimento no sistema de vazão do percolador ou mesmo transpor a membrana filtrante do percolador e liberar resíduo sólido para o extrato (< 710 µm.). Para isso, diversos sistemas de moagem foram testados, sendo a moagem por moinho de martelo refrigerado ou através de um *blender* semi-industrial de alta performance, os sistemas mais eficazes e ágeis. Em seguida, para separar as partículas com granulometrias ideais foram utilizados tamises com diferentes tamanhos de malhas (0,5 – 0,8 mm). O pó obtido foi caracterizado seguindo metodologias descritas na farmacopeia brasileira para a caracterização de matérias-primas vegetais. A amostra apresentou umidade de 17,55 ± 0,39 g/100g, total de cinzas de 5,30 ± 0,09 g/100g, índice de intumescência de 2,1 ± 0,1 mL, atividade de água igual a 0,7± 0,0 e perfil granulométrico moderadamente grosseiro (>710 mm).

Em seguida, concentrações de etanol e água no solvente de extração e diferentes proporções do pó do fruto do café para o solvente foram estudadas a fim de obter um maior rendimento do processo e maior conteúdo de bioativos no extrato. A definição desses parâmetros foi baseada no resultado obtido pelo planejamento experimental delineamento composto central rotacional (DCCR), utilizando a superfície de resposta. Após a obtenção do extrato fluido concentrado com as seguintes características físico-químicas: conteúdo de resíduo sólido de 14,2% (m/m), 3,7% de etanol (v/v), densidade relativa de 1,05 g/mL a 20ºC e viscosidade de 19,65 mPAs a 28ºC, o extrato foi seco pelo método de secagem por aspersão ou por sublimação em liofilizador. O extrato otimizado, seco por liofilização, apresentou o conteúdo de 9750,89±274,15 de 5-*O*-CQA, de 6822,05±126,99 para cafeína e 2663,96±118,48 mg/100g b.s. de trigonelina.

Utilizando os mesmos parâmetros otimizados para extração em pequena escala, foram desenvolvidos em larga escala extratos de café arábica e café Robusta. Os pós foram obtidos pelo método de secagem por aspersão. Nestes, os conteúdos encontrados de ácidos clorogênicos totais (soma deácido 3-*O*-cafeoilquinico, 4-*O*-cafeoilquinico e 5-*O*-cafeoilquinico) foram de 21703,1±408,1 mg/100g b.s. no extrato de café Robusta e de 13437,7±189,9 mg/100g b.s. no extrato obtido do fruto do café arábica. Assim como o conteúdo de ácidos clorogênicos totais, o conteúdo de cafeína no extrato de café Robusta também foi mais elevado (8746,41±204,8

mg/100g b.s.) que no café arábica (6991,03±77,78 mf/100g). Dos compostos bioativos quantificados, somente a trigonelina foi encontrada em maior quantidade no café arábica, cujo valor foi de 5236,09±68,20 mg/100g b.s. enquanto que no extrato obtido do café Robusta o conteúdo encontrado foi de 3557,22±124,70 mg/100g bs.

Para o encapsulamento do extrato fluido obtido, foram estudados métodos já propostos anteriormente na literatura, mas nunca antes aplicados ao extrato obtido do fruto do café verde. A técnica de microencapsulação tem sido amplamente empregada no setor alimentício e farmacêutico como forma de melhorar a estabilidade de ingredientes funcionais como vitaminas, antioxidantes, bioativos, corantes, entre outros produtos. Em relação ao extrato do café obtido, por conta do alto conteúdo de polifenóis e cafeína, para que este produto possa ser empregado na suplementação de alimentos e bebidas o emprego da técnica de microencapsulação é essencial, tendo em vista que os polifenóis formam complexos com proteináceos diminuindo a biodisponibilidade destes macronutrientes presentes na matriz alimentícia. Além de alterar o valor nutricional do alimento, esta complexação altera a capacidade antioxidante dos polifenóis e pode também alterar as características organolépticas do alimento suplementado. Para o desenvolvimento das micropartículas, o extrato fluído concentrado foi incorporado em uma dispersão contendo o polissacarídeo maltodextrina (MD) e o heteropolissacarídeo goma arábica (GA) para servir como material de parede do extrato, e, posteriormente, seco pelo método de secagem por aspersão. A viscosidade e a estabilidade em relação a homogeneidade do sistema dispersivo contendo água, materiais de parede (MD+GA) e o extrato fluido foram as características analisadas para delimitar as proporções de cada ingrediente, para determinar a ordem de incorporação das matérias-primas na produção da dispersão e o tempo e a velocidade de agitação do sistema dispersivo em ultra-turrax.

Apesar das dificuldades encontradas, principalmente na obtenção do pó do fruto do café verde com as características ideias para extração e no desenvolvimento do sistema dispersivo para a microencapsulação, o desenvolvimento do processo de extração e microencapsulação do extrato obtido trouxe avanços ao conhecimento no que se refere a obtenção destes produtos do fruto do café verde, visto que não existem reportados na literatura ou mesmo patenteados, processos de obtenção de micropartículas contendo extrato fluido hidrossolúvel do fruto integro do café verde. Outro avanço obtido neste estudo frente aos extratos já desenvolvidos a partir dos grãos de café cru ou mesmo do fruto integro no estágio cereja de maturação foi a não aplicação de aquecimento em nenhuma das etapas do processo extrativo. Além disso, com o uso do fruto verde há menores risco de contaminação do extrato por micotoxinas frente o uso

de frutos cereja uma vez que a presença de altas quantidades de compostos fenólicos no fruto imaturo diminui ou mesmo impede o crescimento de fungos, de acordo com a literatura.

Os parâmetros estudados no desenvolvimento de um sistema de encapsulação eficiente e com bom rendimento foram: a aplicação de diferentes proporções de goma arábica e maltodextrina na dispersão carreadora e diferentes proporções do extrato fluído em relação ao conteúdo de dispersão. Nove diferentes micropartículas foram obtidas, e aquela com melhores características de estabilidade, rendimento e retenção do marcador químico do extrato, o ácido 5-*O*-cafeoilquínico (5-CQA), bem como o extrato seco não encapsulado otimizado para o maior conteúdo de 5-CQA, foram aplicados em estudos de atividade antioxidante em óleo de girassol, armazenado em condições de temperatura e umidade extremas, e estudos de segurança *in vivo*.

Anteriormente aos estudos de atividade antioxidante e análises de toxicidade *in vivo,* os compostos majoritários encontrados no extrato do café foram avaliados via simulação computacional utilizando as ferramentas ACD/I-Lab, AdmetSAR, e pKCSM que fornecem dados preditivos de toxicidade dos compostos químicos. Os dados apresentados por estas ferramentas indicam que os principais compostos encontrados no extrato (5-CQA, cafeína, trigonelina, ácido cafeico e ácido *p*-coumárico) apresentam baixo risco de toxicidade, sendo a cafeína o composto que apresentou a menor dose letal 50 (DL_{50}), ou seja, a dose que mata 50% do grupo de indivíduos testados. A cafeína, através da ferramenta ACD/I-Lab, apresentou a DL_{50} via oral de 370 mg/kg p.c. enquanto que a trigonelina, ácido cafeico, 5-CQA e ácido *p*-coumarico apresentaram 510, 2100, 2500 e 2500 mg/kg p.c., respectivamente. Os mesmos parâmetros otimizados para o desenvolvimento do extrato de café Robusta microencapsulado, foi utilizado para o desenvolvimento de micropartículas contento extrato de café arábica, obtido também nas mesmas condições de extração previamente otimizadas.

Para testar a eficiência do extrato não encapsulado e das micropartículas como agente antioxidante em matriz alimentícia, o óleo de girassol foi o escolhido devido ao maior conteúdo de ácidos graxos insaturados (91%) comparado a outros óleos vegetais. Tanto o extrato de café arábica quanto o de café Robusta foram testados como agente antioxidante. Ambos produtos, micropartículas e extrato não encapsulado, foram padronizados para conter 0,04% de 5-CQA, conteúdo de 5-CQA isolado previamente testado em outro estudo como agente antioxidante em óleo de soja. Neste ensaio, as micropartículas apresentaram maior eficiência antioxidante frente aos extratos livre e ao antioxidante sintético butil hidroxitolueno (BHT), amplamente empregado na indústria alimentícia. Tem se especulado que este resultado se deve a liberação prolongada dos ativos antioxidante contidos nas micropartículas, enquanto que os extratos livres podem funcionar como pró-oxidante devido ao alto conteúdo de ácidos fenólicos

disponíveis uma vez que estes compostos podem estimular a formação do radical hidroxila pela reação de Fenton na presença de metais de transição, ou mesmo podem funcionar como pró-oxidantes no estado reduzido. Mesmo diante desta possibilidade, os extratos não encapsulados mostraram ser significativos na redução da formação dos produtos secundários da oxidação, mensurados pelo método do ácido tiobarbitúrico (TBAR), quando comparados com as amostras controle, ou seja, sem adição de antioxidantes e amostras contendo micropartículas vazias, sem adição do extrato.

Outra contribuição relevante deste trabalho foi o estudo do perfil de segurança do extrato de café Robusta não encapsulado e microencapsulado, utilizando animais de laboratório nos testes de toxicidade aguda e toxicidade subaguda. Além disso, a possível propriedade estimulante do extrato não encapsulado foi avaliada por estudo comportamental pelo método *open-field*, amplamente aplicado nesse tipo de avaliação. Tanto as micropartículas quanto o extrato não encapsulado foram calculados para apresentar conteúdos similares de bioativos utilizando o fator de correção igual a 3,5 que está ligado ao maior conteúdo de 5-CQA, cerca de 3 partes e ½, no extrato não encapsulado em comparação as micropartículas. No estudo de toxicidade aguda, realizados com camundongos machos e fêmeas, doses únicas administradas oralmente de 0, 100, 500 e 1000 mg/kg p.c. não causaram nenhum efeito adverso em ambos os sexos, no entanto, na dose de 2500 mg/kg p.c do extrato não encapsulado, os animais apresentaram inicialmente sinais autonômicos de agressividade, reação de fuga aumentada, taquipnéia e agitação e posteriormente sinais de ataxia e ptose palpebral, efeitos que podem estar associados a intoxicação por cafeína. Esses efeitos não foram observados nos animais que receberam o extrato encapsulado. A DL_{50} do extrato não encapsulado foi encontrada na dose de 5000 mg/kg p.c., na qual 66,6% dos machos e 50% das fêmeas vieram a óbito. Já a DL_{50} das micropartículas não foi determinada neste estudo, visto que a dose de 5000 mg/kg p.c. não causou a morte de nenhum animal. Como visto anteriormente no estudo computacional *in silico*, a DL_{50} da cafeína é menor que a de outros compostos majoritários quantificados no extrato. Desta forma, o efeito toxico agudo do extrato não encapsulado pode ser atribuído a cafeína visto que na dose mais alta, o conteúdo de extrato administrado fornece uma quantidade de cafeína suficiente para causar uma overdose. O mesmo efeito não foi observado nas micropartículas presumivelmente devido ao fato da liberação controlada *in vivo* do conteúdo de cafeína e outros compostos que podem vir a potencializar o efeito da mesma, presentes no extrato de café verde, promovida pelos materiais de parede.

No estudo comportamental, somente a dose de 1000 mg/kg p.c. após 30 minutos de administração oral do extrato não encapsulado aumentou significativamente a agitação dos camundongos machos, medida pelo número de cruzamentos e número de levantadas ou *rearing* (ficar em duas patas) dos camundongos. Entretanto, a dose de 2000 mg/kg p.c. diminuiu a agitação comparado ao grupo que recebeu 1000 mg/kg p.c., mas não em comparação ao grupo controle em relação ao número de cruzamentos. Já em relação ao número de *rearing*, este grupo apresentou valores significativamente menores em relação ao controle após 30 e 60 minutos de ter recebido o extrato via oral. Esse comportamento observado na dose mais alta pode ter ocorrido devido a algum desconforto sentido pelos animais, tal como náusea, tontura, hipotensão, entre outros efeitos reportados na intoxicação por cafeína.

Para o estudo de toxicidade subaguda, ratos machos, pesando entre 180 e 220 gramas, receberam durante 30 dias doses de 0, 100, 500 e 1000 mg/kg p.c. Nenhum dos grupos testados apresentou efeitos adversos ou morte durante o tempo de tratamento, ambos apresentaram eficiência alimentar, consumo de água, consumo de ração e ganho de peso similares ao grupo controle. Não houve alterações bioquímicas ou hematológicas significativas e/ou condizentes com os resultados encontrados na medição do peso relativo e/ou absoluto dos órgãos, ou mesmo com as análises macroscópica e histológicas dos órgãos selecionados (cérebro, coração, pulmões, rins, fígado, baço, estômago e testículos). Frente a estes achados, a NOAEL (no-observed-effect-adverse level) atribuída ao EFCV microencapsulado é de 1000 mg/kg p.c./dia, e, a dose equivalente calculada para o consumo humano é de 189 mg/kg p.c./dia ou 11,34 gramas diários para um adulto de 60 quilos de massa corporal. O que indica alta margem de segurança no consumo do extrato microencapsulado.

Devido à alta margem de segurança do extrato e micropartículas assim como a estabilidade e funcionalidade dos mesmos, ambos podem ser recomendados como fonte de antioxidante e cafeína que podem ser aplicados para suplementar diversos produtos alimentícios, assim como pode ser consumido de forma isolada como suplemento nutracêutico em diferentes formas farmacêuticas e/ou alimentícias. Ou mesmo, podem ser aplicados como ingrediente na indústria cosmética.

Neste sentido, a patente referente aos processos de obtenção do extrato e micropartículas foi reivindicada junto ao Instituto Nacional de Propriedade Intelectual (INPI) sob o número de pedido BR 10 2017 025848 3.

CAPITULO VIII

CONCLUSÃO GERAL

O método de extração desenvolvido neste estudo para o pó do fruto inteiro do café verde, frente aos existentes para os grãos dos frutos crus e frutos íntegros no estágio cereja apresenta a vantagem de utilizar somente solventes considerados de baixa toxicidade aos seres vivos e ao meio ambiente, como etanol e água, além de não necessitar de uso de calor em nenhuma etapa do processo de extração, diferente de outros métodos reportados na literatura. O método de quantificação e identificação de compostos bioativos tanto no extrato obtido quanto nas micropartículas se apresentou preciso e exato.

O processo de microencapsulação não contribuiu para melhorar a estabilidade da maioria dos compostos analisados, exceto o ácido cafeico, visto que o marcador 5-CQA, a cafeína e a trigonelina se mantiveram estáveis inclusive no extrato não encapsulado. No entanto, a aplicação de 80% de maltodextrina e 20% de goma arábica assim como a proporção de 1:3,5 (m/m, relação de extrato fluido: dispersão) mostrou influência positiva no rendimento do processo, na retenção do 5-CQA durante o processo de secagem por aspersão e na estabilidade do ácido cafeico durante o armazenamento das micropartículas em estufa a 40 ± 5ºC por 180 dias.

Os resultados obtidos neste estudo mostram que o extrato do fruto do café em sua forma microencapsulada é eficaz como antioxidante em alimentos ricos em ácidos graxos insaturados, e, mesmo o extrato não encapsulado, se mostrou tão eficiente quanto o antioxidante sintético em retardar o processo de formação de produtos secundários da oxidação.

Além disso, os estudos de segurança desenvolvidos, tanto *in vivo* quanto por meio de ferramentas computacionais *in silico*, mostraram que o extrato de café verde não encapsulado apresenta alta margem de segurança, pois a DL_{50} do extrato foi definida em 5000 mg/kg p.c. dose na qual, de acordo com a Organisation for Economic Co-operation and Development (OECD), são inseridas as substâncias com risco de toxicidade aguda relativamente baixa. Como a DL_{50} do extrato encapsulado não foi definido mesmo na dosagem de 5000 mg/kg p.c., pode-se considerar que o processo de microencapsulação aumentou ainda mais a margem de segurança do extrato. A NOAEL de 1000 mg/kg p.c., estabelecida para as micropartículas pelo método de toxicidade subaguda, indica que este produto é seguro para ser adicionado em diversos produtos alimentícios aditivo ou suplemento alimentar, ou mesmo para ser consumido puro em formas farmacêuticas como comprimido, drágeas, cápsulas, entre outros. Sendo que a dose equivalente calculada para consumo humano é de 189 mg/kg p.c. dia.

CAPÍTULO IX

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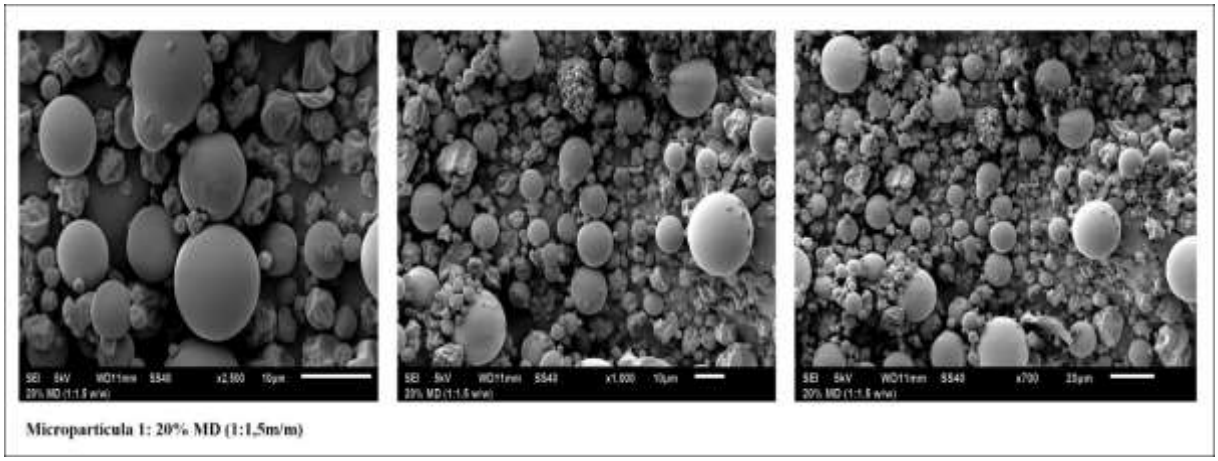
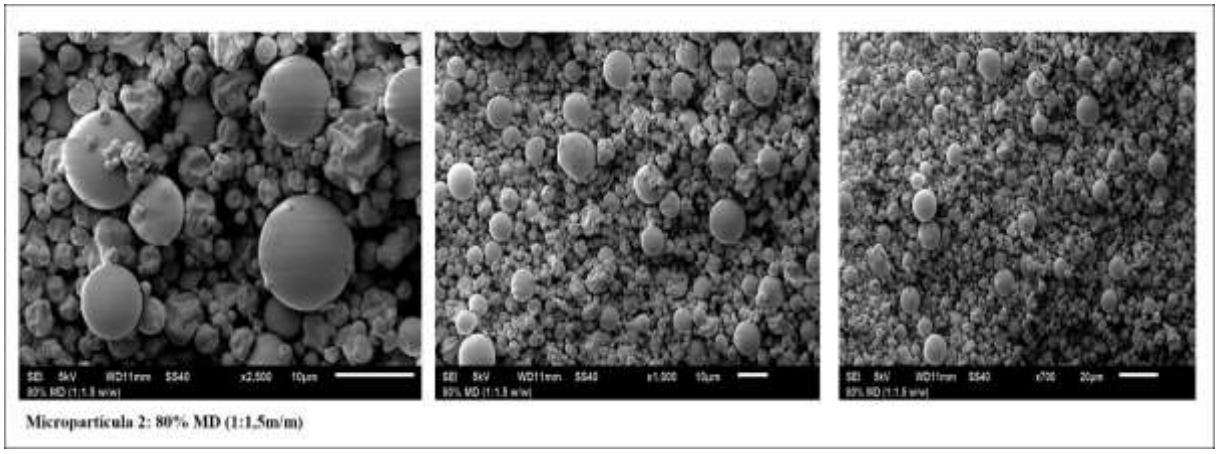
CAPITULO X

APÊNDICE

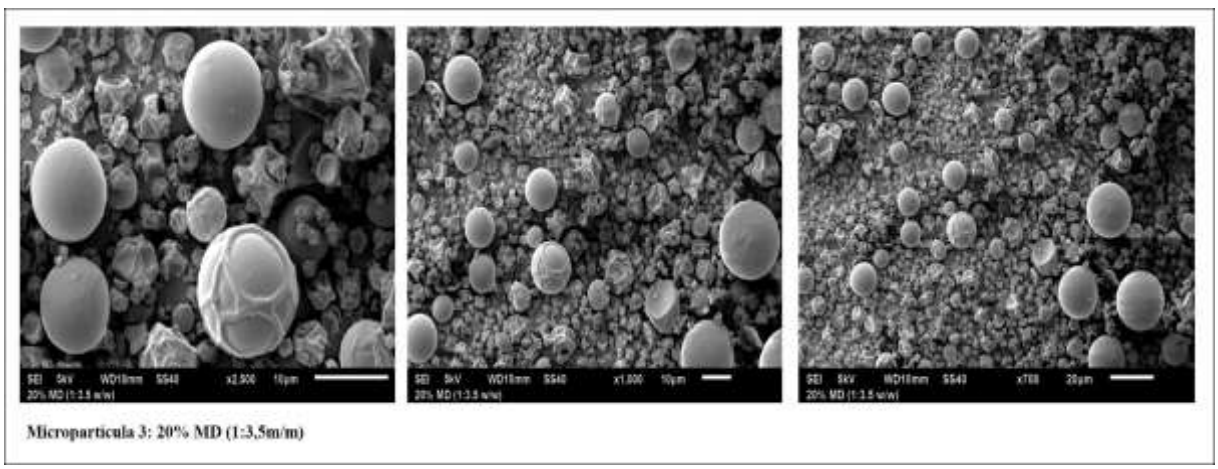
APÊNDICE A. Fotomicrografias obtidas por microscopia eletrônica de varredura (MEV) das micropartículas contendo extrato do fruto do café verde em 2500, 1000 e 700 x 5 kV. E do extrato não encapsulado e micropartículas vazias (controle) em 2500, 1000 e 500 x 5 kV.



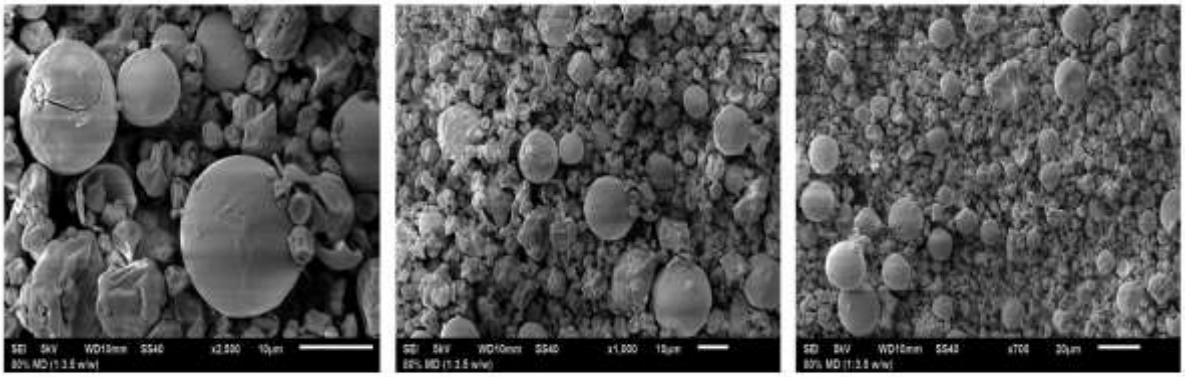
Microparticula 1: 20% MD (1:1,5m/m)



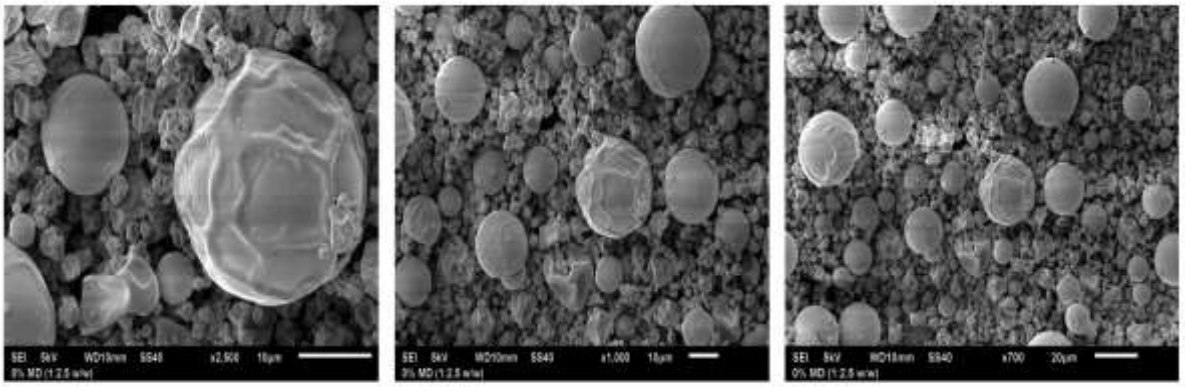
Microparticula 2: 80% MD (1:1,5m/m)



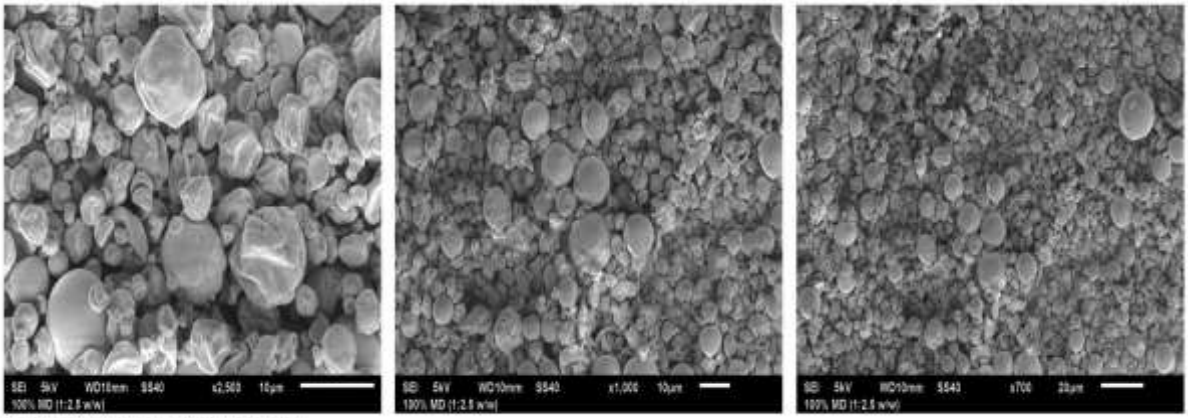
Microparticula 3: 20% MD (1:3,5m/m)



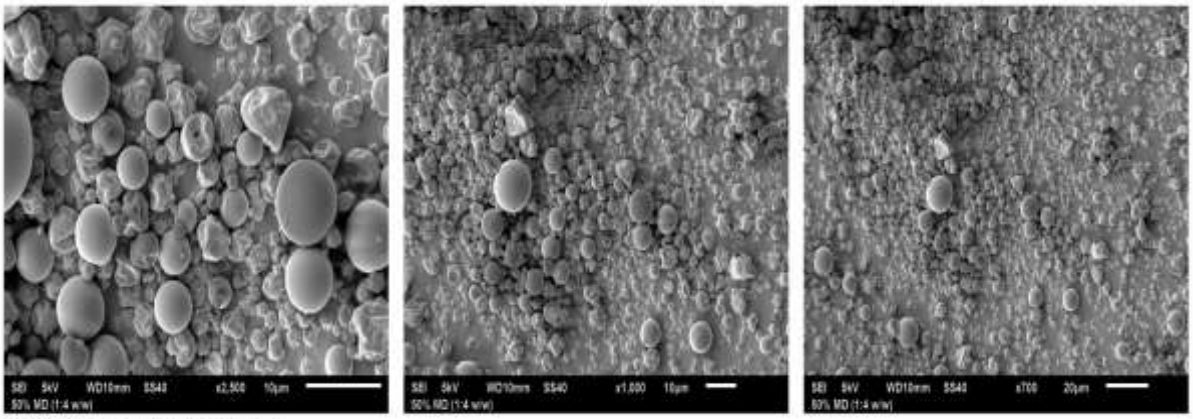
Microparticula 4: 80% MD (1:3,5m/m)



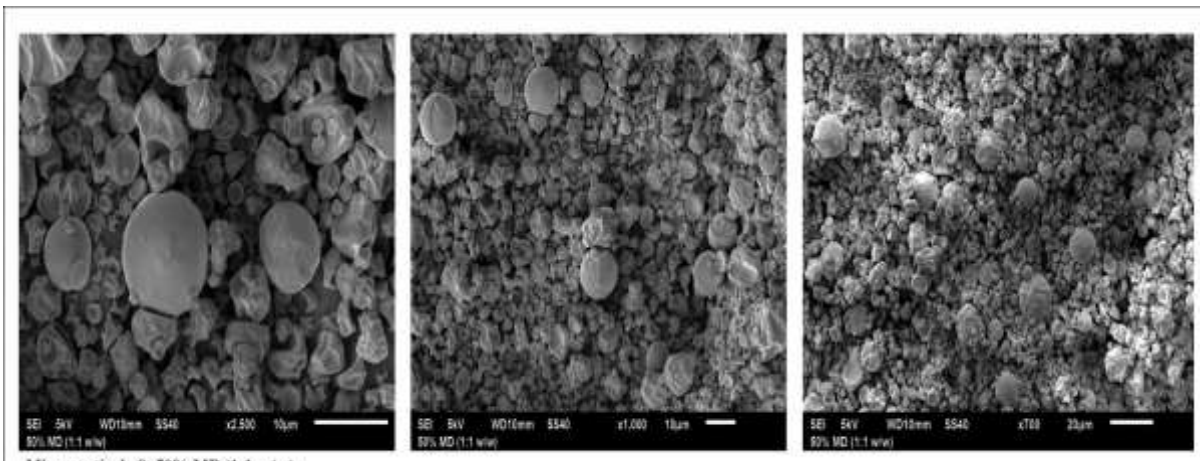
Microparticula 5: 0% MD (1:2,5m/m)



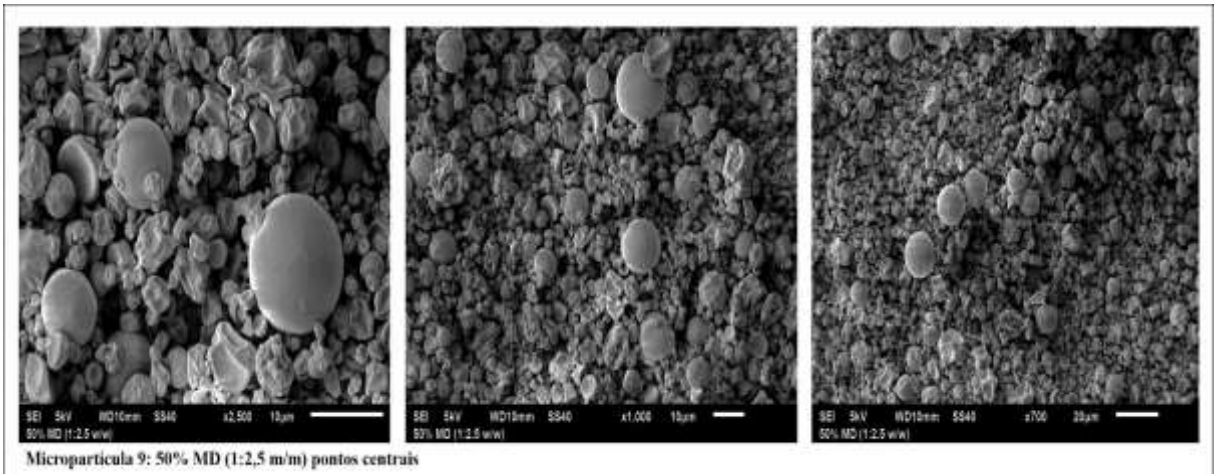
Microparticula 6: 100% MD (1:2,5 m/m)



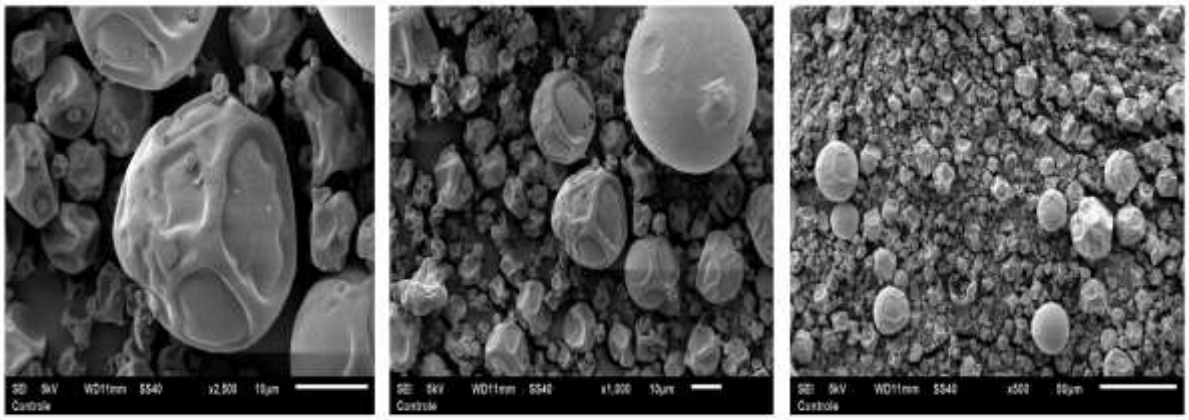
Microparticula 7: 50% MD (1:4 m/m)



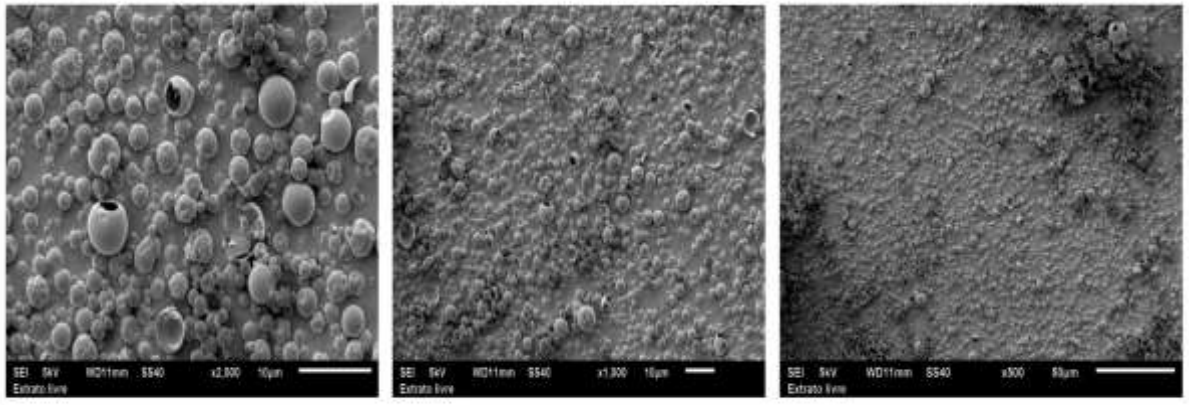
Microparticula 8: 50% MD (1:1 m/m)



Microparticula 9: 50% MD (1:2,5 m/m) pontos centrais

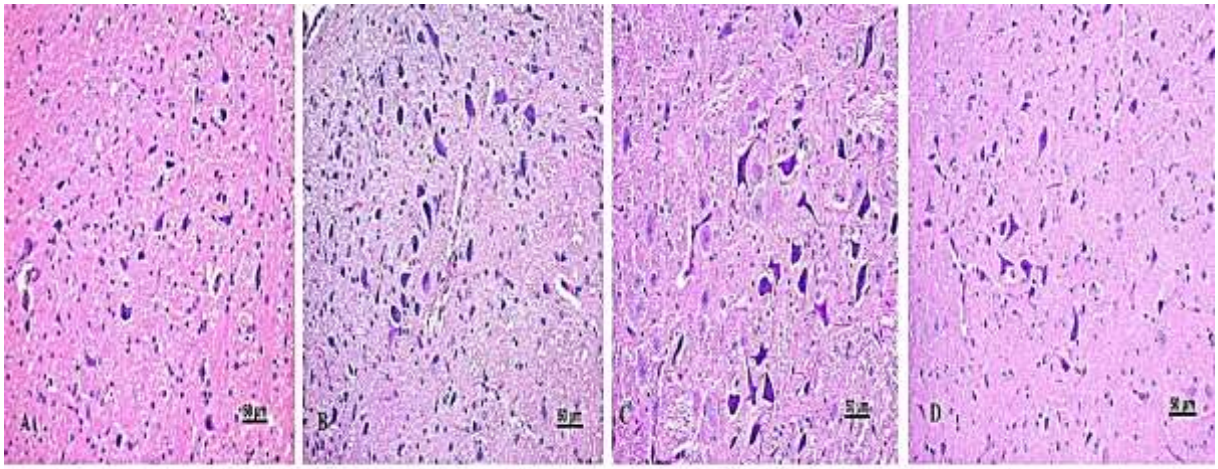


Microparticulas vazias: 50% MD (1:1 m/m)

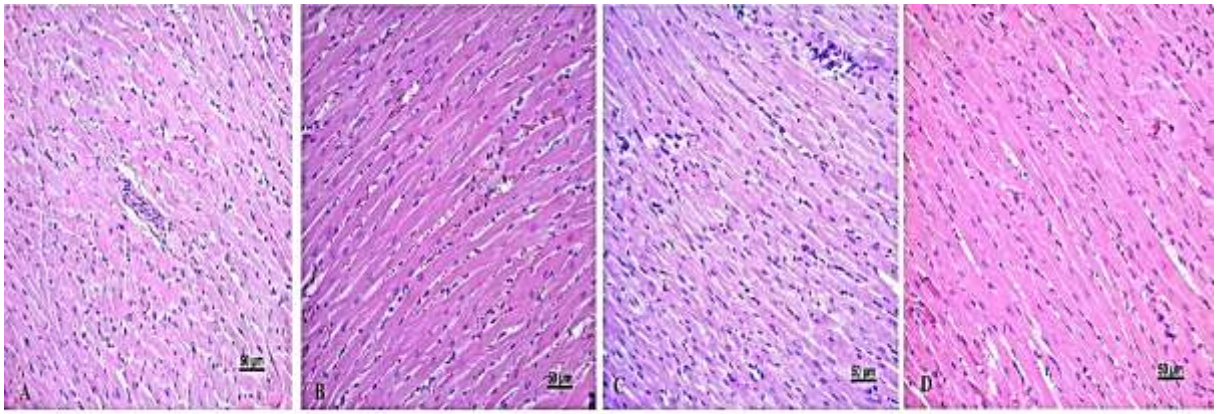


Extrato não encapsulado

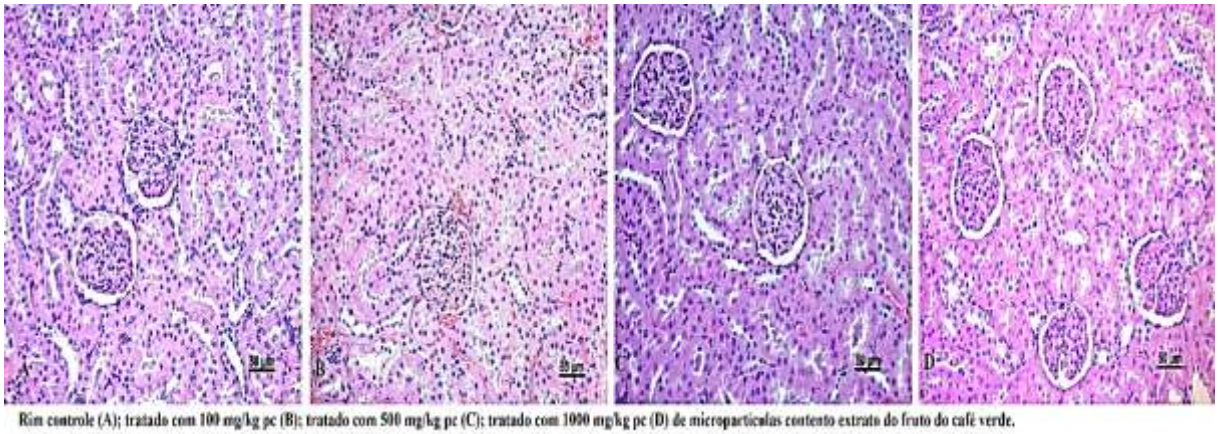
APÊNDICE B. Fotomicrografias em 40x obtidas por microscopia óptica das lâminas histológicas do cérebro, coração, rins, fígado, pulmões, testículos, estômago, baço e intestíno dos ratos controle e tratados durante 30 dias com micropartículas contendo extrato do fruto do café verde.



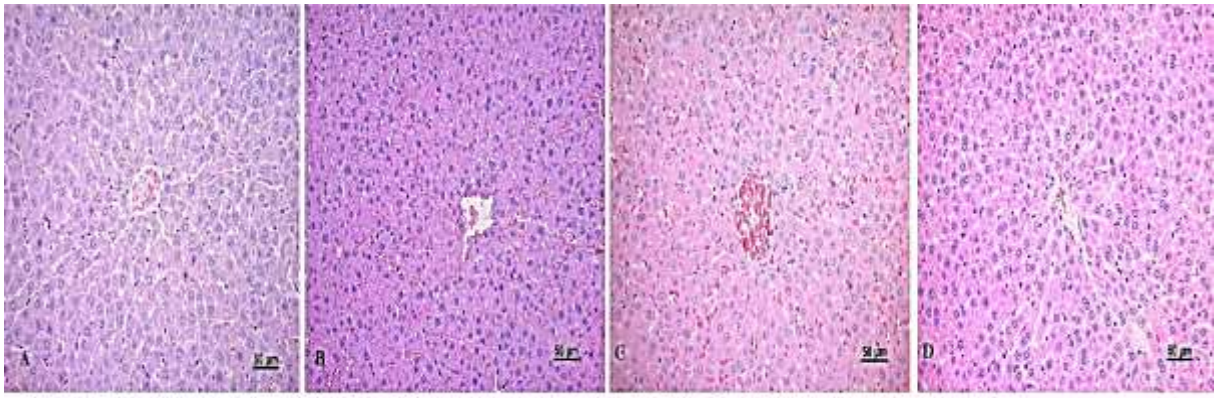
Cérebro controle (A); tratado com 100 mg/kg pc (B); tratado com 500 mg/kg pc (C); tratado com 1000 mg/kg pc (D) de microparticulas contento extrato do fruto do café verde.



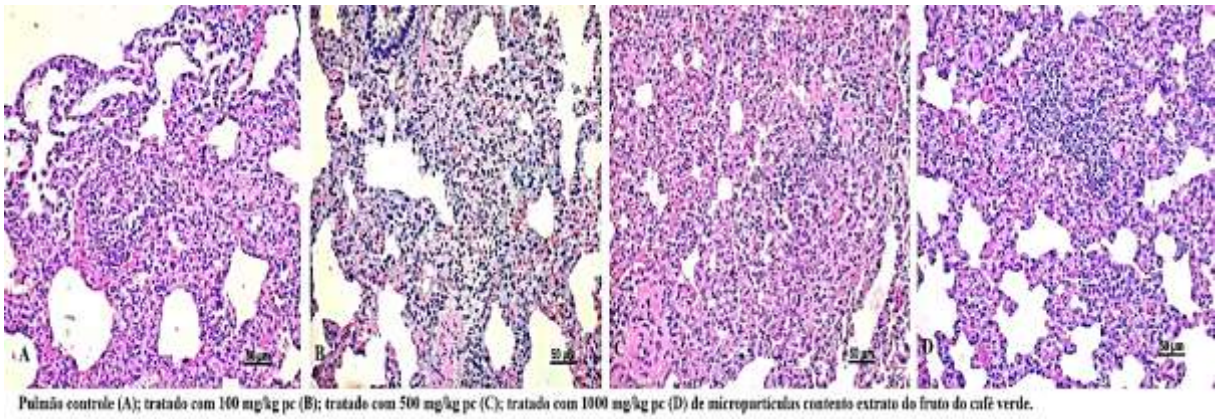
Coração controle (A); tratado com 100 mg/kg pc (B); tratado com 500 mg/kg pc (C); tratado com 1000 mg/kg pc (D) de microparticulas contento extrato do fruto do café verde.



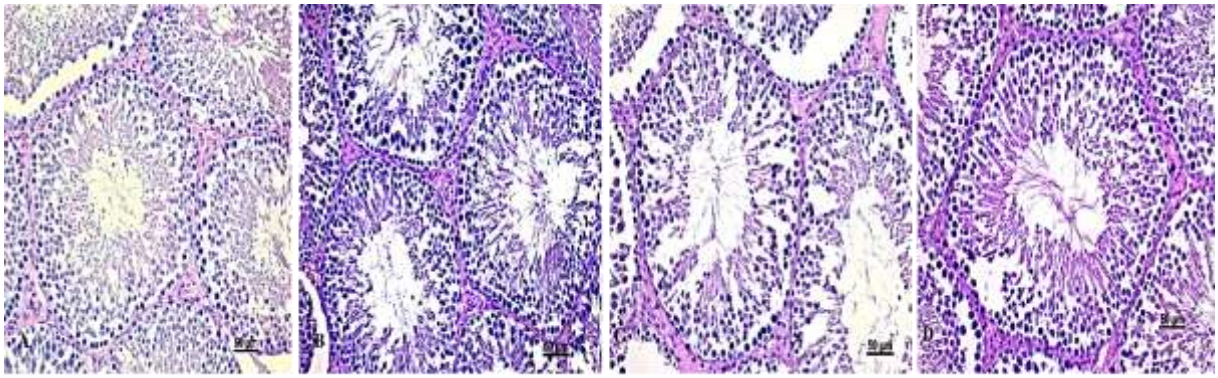
Rim controle (A); tratado com 100 mg/kg pc (B); tratado com 500 mg/kg pc (C); tratado com 1000 mg/kg pc (D) de microparticulas contento extrato do fruto do café verde.



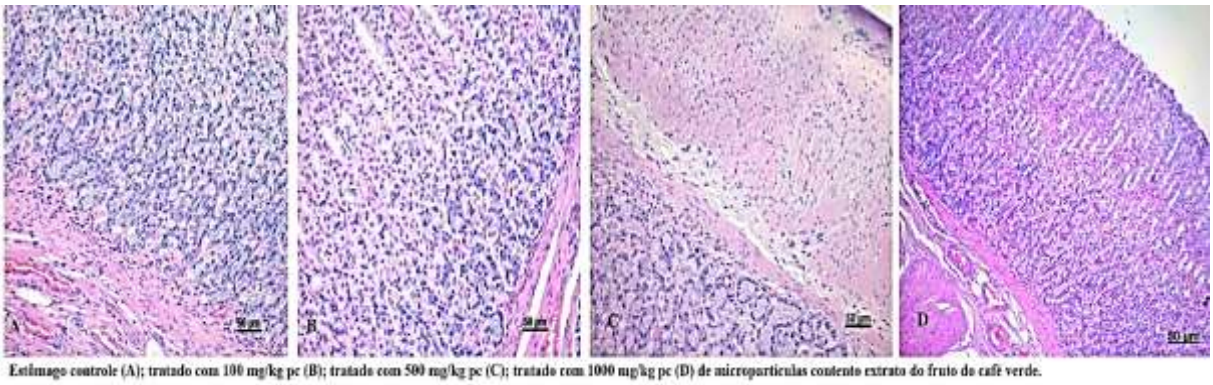
Figado controle (A); tratado com 100 mg/kg pc (B); tratado com 500 mg/kg pc (C); tratado com 1000 mg/kg pc (D) de microparticulas contento extrato do fruto do café verde.



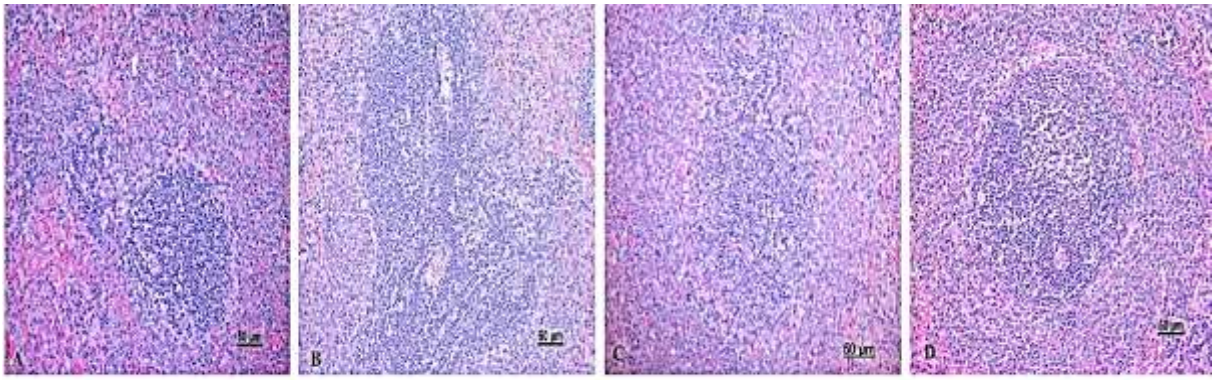
Pulmão controle (A); tratado com 100 mg/kg pc (B); tratado com 500 mg/kg pc (C); tratado com 1000 mg/kg pc (D) de microparticulas contento extrato do fruto do café verde.



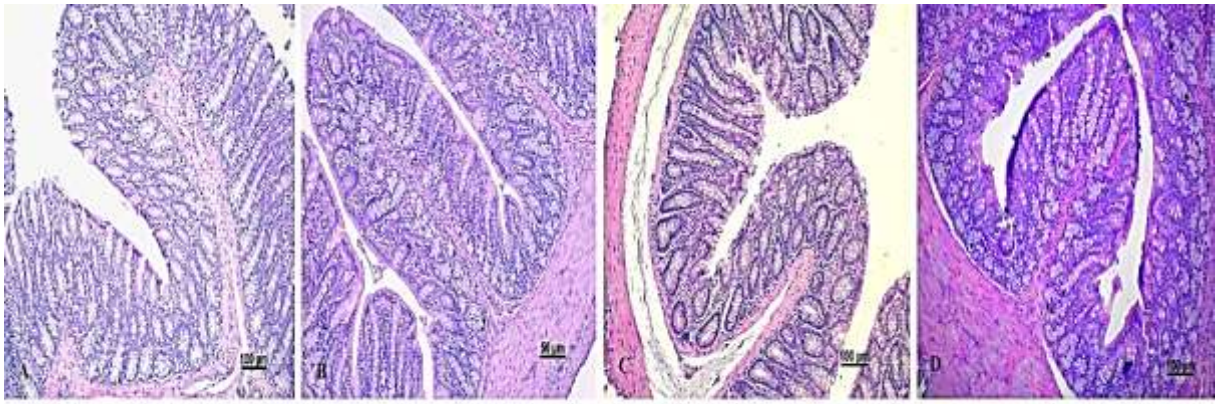
Testículos controle (A); tratado com 100 mg/kg pc (B); tratado com 500 mg/kg pc (C); tratado com 1000 mg/kg pc (D) de microparticulas contento extrato do fruto do café verde.



Estômago controle (A); tratado com 100 mg/kg pc (B); tratado com 500 mg/kg pc (C); tratado com 1000 mg/kg pc (D) de microparticulas contento extrato do fruto do café verde.



Baço controle (A); tratado com 100 mg/kg pc (B); tratado com 500 mg/kg pc (C); tratado com 1000 mg/kg pc (D) de microparticulas contento extrato do fruto do café verde.



Intestino controle (A); tratado com 100 mg/kg pc (B); tratado com 500 mg/kg pc (C); tratado com 1000 mg/kg pc (D) de microparticulas contento extrato do fruto do café verde.

CAPITULO XI

ANEXO

UNIVERSIDADE FEDERAL DE MATO GROSSO

COMITÊ DE ÉTICA NO USO DE ANIMAIS

CEUA

COMITÊ DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO

Certificamos que o Protocolo Nº 23108.101038/2015-91, sobre "Obtenção, caracterização físico-química e estudo de segurança de microcápsulas contendo extrato de café verde (*Coffea arábica*) padronizado em ácido clorogênico", sob a responsabilidade de **Profª Drª NEURA BRAGAGNOLO/**Wanessa Costa Silva Faria & Col., está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), tendo sido aprovado pelo Comitê de Ética no Uso de Animais (CEUA)-UFMT em reunião ordinária de **02/10/2015**.

CERTIFICATE

We certify that the protocol Nº 23108.101038/2015-91, entitled "Development, characterization physicochemical and safety study of microcapsules containing green coffee extract (*Coffea arábica*) standardized in chlorogenic acid", is in agreement with the Ethical Principles for Animal Research established by the National Council for Control of Animal Experimentation (CONCEA). This project was approved by the institutional Committee for Ethics in the Use of Animals (Federal University of Mato Grosso – UFMT) on **October 02, 2015**.

Cuiabá-MT, 02 de outubro de 2015.

Prof. Dr. Roberto Vilela Veloso
Presidente

Prof. Dr. Daniel Moura de Aguiar
Vice-Presidente

Universidade Federal de Mato Grosso – UFMT
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Data: sexta-feira, 1 de dezembro de 2017 08:45:13 BRST

Prezados Inventores:
É com satisfação que informamos que o pedido de patente abaixo foi devidamente depositado junto ao Instituto Nacional de Propriedade Industrial
(INPI).
Seguem os dados e também cópia em pdf do processo integral.
A área de parcerias se dedica em buscar um parceiro que possa explorar as
patentes e PCs. Caso tenha algum possível interessado em mente, contate a
parcerias através de parcerias@inova.unicamp.br. A diretora é a Iara Ferreira.

Número: BR 10 2017 025848 3
Data de Protocolo: 30.11.17
Título: PROCESSO DE OBTENÇÃO DE MICROCÁPSULAS DE CAFÉ VERDE,
MICROCÁPSULAS DE CAFÉ VERDE ASSIM OBTIDAS E USO

Fase: Patente requerida
Parabéns a toda equipe

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