UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ENGENHARIA DE ALIMENTOS

GUILHERME COELHO LOPES DOS REIS

AMINAS BIOATIVAS E AMINOÁCIDOS NO COGUMELO *Agaricus bisporus*: CARACTERIZAÇÃO, PROCESSAMENTO E BIOACESSIBILIDADE

BIOACTIVE AMINES AND AMINO ACIDS IN *Agaricus bisporus* MUSHROOM: CHARACTERIZATION, PROCESSING AND BIOACCESSIBILITY

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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 Doutor em Ciência de Alimentos.

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“Eu vou lhe explicar o que é um bom pesquisador. Ele é um bom profissional, é um homem que tem uma profissão e a exerce com dignidade e competência. Ele não é Deus, ele não é sábio, ele não é nada! Ele é um profissional.”

(Paulo Vanzolini)

RESUMO

Este trabalho teve como objetivo a investigação da influência do processamento (cozimento e conserva) na bioacessibilidade de aminoácidos e aminas bioativas em cogumelos *Agaricus bisporus*. Para tal, foi otimizado um método para análise simultânea de aminoácidos e aminas bioativas livres em cogumelos com derivatização pré-coluna com carbamato de 6-aminoquinolil-N-hidroxysuccinimidil (Accq), separação por UPLC e detecção no UV (249 nm). A extração destes analitos foi otimizada usando delineamentos Plackett-Burman e composto central rotacional (DCCR). O método otimizado foi validado de acordo com as diretrizes da Comunidade Europeia referente a norma 657/2002 e foi rápido, fácil, preciso, sensível e confiável, adequado ao propósito. O cogumelo *Agaricus bisporus* (n=9) foi caracterizado pela presença de 15 aminoácidos livres, apresentando o teor total de aminoácidos livres igual a 470,0 mg/100g e apenas uma amina – espermidina foi detectada (7,8 mg/ 100g). Alanina, ácido glutâmico e prolina foram os aminoácidos livres predominantes nesses cogumelos. O processamento doméstico – cozimento, não afetou o conteúdo de espermidina, entretanto houve perda significativa (14 a 23%) para o produto em conserva. Em relação aos aminoácidos totais, houve uma perda média de 39,7% no cozimento e 64,6% na conserva. As perdas de aminoácidos variaram em função do tratamento. O processamento não afetou o percentual de hidrólise proteica dos cogumelos. Análises de componentes principais e agrupamentos hierárquicos mostraram que a hidrólise de proteínas nos cogumelos processados é muito pequena na fase gástrica. O teor de espermidina permaneceu o mesmo após a digestão *in vitro*. Embora os cogumelos apresentem baixo teor de proteínas, o processo de digestão é capaz de liberar grande parte dos aminoácidos da proteína. Os dados de bioacessibilidade de espermidina em *A. bisporus*, podem aumentar o valor biológico deste alimento.

Palavras chave: Espermidina, UHPLC, ácido glutâmico, digestão *in vitro*.

ABSTRACT

This work aimed to investigate the influence of processing (cooking and preserving) on the bioaccessibility of amino acids and bioactive amines in mushrooms. For this purpose, a method for simultaneous analysis of free bioactive amino acids and amines in mushrroms with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (Accq) pre-column derivativation, UPLC separation and UV detection (249 nm) was optimized. Extraction of these analytes was optimized using Plackett-Burman designs and central rotational compound (DCCR). The optimized method has been validated according to European Community guidelines for 657/2002 and was fast, easy, accurate, sensitive and reliable, fit for purpose. The mushroom *Agaricus bisporus* (n = 3) was characterized by the presence of 15 free amino acids, with a total free amino acid content of 470.0 mg / 100g and only one amine - spemidine was detected (7.8 mg / 100g). Alanine, glutamic acid and proline were the free amino acids predominant in mushrooms. Domestic processing - cooking did not affect spermidine content, however there was a significant loss (14 to 23%) for the canned process. Regarding total amino acids, there was an average loss of 39.7% in cooking and 64.6% in canned. Amino acid losses varied with treatment. Processing did not affect the percentage of *in vitro* protein hydrolysis of mushrooms. Principal component analyzes and hierarchical clusters showed that protein hydrolysis in processed mushrooms is very small in the gastric phase. The spermidine content remained the same after *in vitro* digestion. Although mushrooms are low in protein, the digestion process is able to release much of the protein's amino acids. The bioaccessibility data of spermidine in *A. bisporus*, can increase the biological value of this this food.

Keywords: Spermidine, UHPLC, glutamic acid, *in vitro* digestion.

SUMÁRIO

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INTRODUÇÃO GERAL

Os cogumelos são alimentos muito apreciados desde a idade antiga por se acreditar em seu elevado valor nutricional e potencial medicinal (Furlani & Godoy, 2007). Existem aproximadamente 2.000 espécies de cogumelos potencialmente comestíveis, das quais apenas 30 são normalmente utilizadas na alimentação humana (Chang & Miles, 2004, Cheung 2010).

No Brasil, as espécies popularmente cultivadas são *Agaricus bisporus, Agaricus blazei, Lentinula edodes* e algumas espécies do gênero *Pleurotus* (Furlani & Godoy, 2007). De acordo com Associação Nacional dos Produtores de Cogumelos (ANPC), o cogumelo *Agaricus bisporus* é o mais produzido. Os cogumelos são geralmente comercializados nas formas *in natura*, desidratado, em conserva, ou em alimentos processados como sopas, molhos e outros. Contudo, o consumo de cogumelos no Brasil ainda é pequeno quando comparado ao consumo em países europeus como Itália, França e Alemanha e em países asiáticos como China e Coreia do Sul (ANPC, 2019).

Além do uso alimentício, o uso medicinal dos cogumelos tem uma longa história em países da Ásia (especialmente China, Coréia e Japão), ao passo que a utilização para esse fim é mais recente no ocidente. A atividade medicinal dos cogumelos é caracterizada pela presença de metabólitos primários e secundários com amplas atividades farmacológicas (Chang & Miles, 2004; Cheung, 2010; Roupas et al., 2012; Feeney et al., 2014; Pop et al., 2018).

As aminas bioativas também estão presentes em cogumelos, tanto as poliaminas quanto as aminas biogênicas. As poliaminas (espermidina e espermina) são fatores de crescimento que ocorrem nas células de organismos vivos onde desempenham diversas funções fisiológicas. Portanto, as poliaminas são úteis em pacientes em pós-operatórios, durante o crescimento ou desenvolvimento do sistema digestivo. (Kalač & Krausová, 2005, Glória, 2005, Kalač, 2014).

Outras aminas bioativas conhecidas como aminas biogênicas podem também ser encontradas nos cogumelos (Yamoto et al., 1982, Yen, 1992; Kalac & Krizek, 1996, Okamoto et al., 1997, Nishimura et al., 2006, Nishibori et al., 2007 Dadaková et al.,

2009). Essas aminas são conhecidas por apresentarem atividade vaso e neuroativa e quando presentes em elevadas quantidades nos alimentos podem causar efeitos adversos a saúde (EFSA, 2011). A histamina e a tiramina são as aminas mais relacionadas aos quadros de intoxicação alimentar. Em indivíduos que fazem uso de alguns fármacos a presença de pequenas quantidades de tiramina no alimento pode levar a crises hipertensivas (EFSA, 2011).

As aminas podem também ser formadas por descarboxilação de aminoácidos livres por enzimas de microrganismos contaminantes e, por serem termoresistentes, as aminas permanecem no alimento, mesmo após tratamento térmico podendo ser utilizadas como indice de qualidade higiênico sanitária em alimentos (Silla-Santos, 1996, Eliassen et al., 2002, Glória 2005; Dadáková et al., 2009, EFSA, 2011; Kalač, 2013, Feeneey et al., 2014, Papageorgiou et al., 2018).

Dessa forma, a determinação de aminoácidos livres em cogumelos também é importante, uma vez que a disponibilidade destes pode refletir no potencial de produção de aminas bioativas (Jia et al., 2011; Fiechter et al., 2013; Redruello et al., 2017). Além de serem precursores para a síntese de aminas bioativas, os aminoácidos são componentes essenciais na dieta humana, indispensáveis para funções vitais uma vez que estão envolvidos na biossíntese de enzimas, hormônios e proteínas estruturais e podem participar ativamente do sabor de um alimento (FAO, 2007, Marchini et al., 2016).

Pelo fato de os cogumelos serem altamente perecíveis, devido a elevada atividade de água quando frescos e vida útil de 5 a 6 dias sob refrigeração, o processamento de cogumelos torna-se necessário para aumentar a vida útil dos mesmos (Sampaio, 2003). Contudo o processamento de cogumelos pode alterar as propriedades nutricionais desse alimento (Chiang et al., 2006; Fei et al., 2007; Tsai et al., 2007; Kim et al., 2009; Jarwoska & Bernaś, 2013; Liu et al., 2014; Rotola-Pukkila et al., 2019), inclusive os teores de aminoácidos e de aminas bioativas. A análise simultânea de aminas bioativas e aminoácidos em cogumelos pode ser uma ferramenta útil para não apenas para fornecer informação nutricional, mas também pode possibilitar o monitoramento da qualidade higiênico-sanitária durante o processamento (Fiechter et al., 2013).

Além do processamento, a investigação da bioacessibilidade ao longo do trato gastrointestinal e a liberação de poliaminas e aminoácidos da matriz do alimento podem ajudar a esclarecer o impacto destes compostos provenientes da dieta a saúde humana.

A bioacessibilidade é definida como a quantidade de um nutriente ingerido que está disponível para absorção no intestino após a digestão. A bioacessibilidade pode ser determinada por métodos *in vivo* e *in vitro.* Os métodos *in vivo* geram dados diretos e envolvem animais e humanos, o que representa alto custo e impedimentos éticos para serem realizados (Guerra et al., 2012; Minekus et al., 2014). Dessa forma a abordagem *in vitro* torna-se preferencial por seu baixo custo e ausência de constrangimentos éticos (Mercadante & Mariutti, 2018).

O estudo da bioacessibilidade *in vitro* simula a passagem de um alimento pelo sistema gastrointestinal. As técnicas *in vitro* melhoram a acurácia e reprodutibilidade e a bioacessibilidade é avaliada pela quantificação dos nutrientes liberados do alimento, nas etapas do processo digestivo ou no processo digestivo como um todo. O processo de digestão é simulado utilizando enzimas digestivas sob condições controladas. A digestão gástrica é estimulada por digestão pepsina-HCl, seguido de pancreatina com sais biliares (digestão no intestino). Os modelos podem ser estáticos ou dinâmicos (Mercadante & Mariutti, 2018). Apesar da variedade de modelos, o estudo de bioacessiblidade é uma alternativa simples e válida para o entendimento da digestão e da influência de algumas variáveis da digestão com relação a funcionalidade do alimento (Ariza et al., 2018; Cilla et al., 2018; Mercadante & Mariutti, 2018)

Dessa forma esse trabalho teve como objetivo geral, caracterizar a composição e a bioacessibilidade de aminoácidos e aminas bioativas em cogumelos *Agaricus bisporus*.

Os objetivos específicos foram,

(i) desenvolver e validar um método analítico para determinação simultânea de aminas bioativas e aminoácidos em cogumelos, utilizando cromatografia líquida de ultra eficiência, com derivatização pre-coluna via reação com 6-aminoquinolil-N-hidroxisuccinimidil (Accq), separação em fase reversa e detecção na região do ultravioleta (249 nm);

(ii) avaliar o efeito do tratamento térmico e da produção de conserva no perfil e teores de aminas bioativas e aminoácidos em cogumelos;

(iii) Avaliar o efeito da digestão *in vitro* com relação ao perfil e teor de aminas bioativas e aminoácidos em cogumelos frescos e processados.

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CAPÍTULO I: REVISÃO BIBLIOGRÁFICA

1. Cogumelos comestíveis

1.1. Definição, produção e consumo

Cogumelos são macrofungos com corpos de frutificação distinguíveis, os quais são largos o suficiente para serem vistos ao olho nu e colhidos manualmente. Podem ter crescimento acima (epigeus) ou abaixo (hipogeus) do solo. O corpo de frutificação, conhecido como cogumelo, é a estrutura reprodutora de um organismo maior, dividido em estruturas vegetativa e reprodutora. A estrutura vegetativa é conhecida como micélio, formado pela fusão de hifas compatíveis que derivam de diferentes esporos liberados pelos corpos de frutificação (estrutura reprodutora) (Figura 1) (Chang & Miles, 2004).

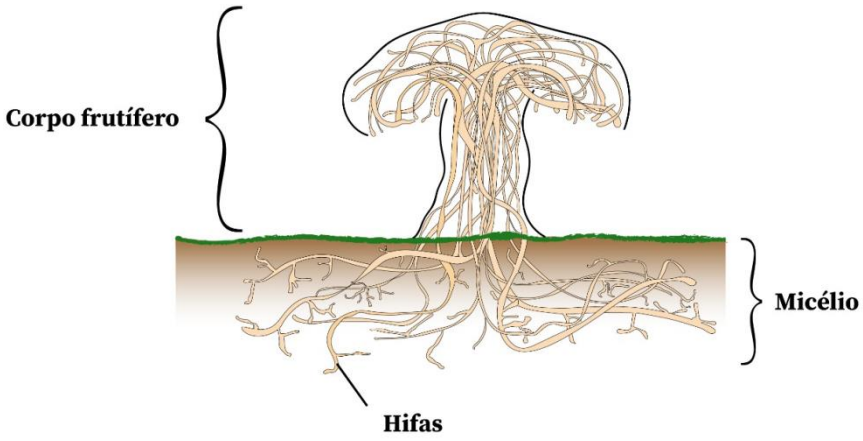

Figura 1. Esquema da estrutura de macrofungos destacando as hifas, o micélio e o corpo de frutificação. Fonte: O autor.

Os cogumelos podem ser classificados em três categorias: comestíveis, medicinais e tóxicos – ou venenosos. Os comestíveis (principalmente os corpos de frutificação) podem ser consumidos tanto frescos, secos ou conservados de outras formas (ex. *Agaricus bisporus, Lentinula edodes*). Os cogumelos medicinais são fungos que não são usados para propósitos culinários, contêm compostos bioativos e têm aplicação medicinal (ex. *Ganoderma lucidum, Agaricus blazei*). Já os cogumelos

venenosos são aqueles que são comprovadamente ou suspeitos de serem venenosos (ex. *Amanita phalloides* ou chapéu da morte) (Cheung, 2010).

O tipo mais familiar dos corpos de frutificação da classe Basidiomycetes apresenta chapéu (píleo), guelra, estipe e, algumas vezes, vulva como no caso do cogumelo da espécie *Volvariella volvacea* (Figura 2) (Chang & Miles, 2004).

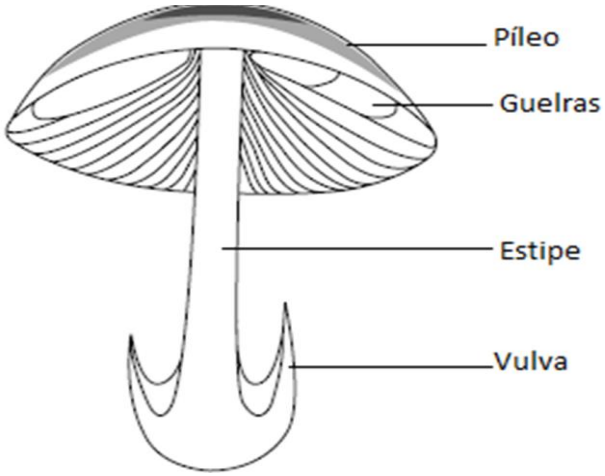

Figura 2. Desenho do cogumelo *Volvariella volvacea*, mostrando o píleo, as guelras, o estipe e a vulva (adaptado de Chang & Miles, 2004).

Os cogumelos selvagens têm feito parte da dieta humana por séculos devido as suas características nutricionais, sensoriais e propriedades medicinais. Alguns cogumelos selvagens foram domesticados sendo cultivados no mundo inteiro. As espécies *Agaricus bisporus*, *Pleurotus ostreautus* e *Lentinula edodes* representam as espécies de cogumelos mais cultivadas no mundo (Royse, 2017). Outras espécies de cogumelos com alto valor econômico como trufa (*Tuber melanosporum*), porcini (*Boletus edules*), cantarelo (*Cantharellus cibarius*) e matsutake (*Tricholoma matsutake*) também são cultivadas.

A produção mundial de cogumelos teve um crescimento de 73% em 10 anos aumentando de 5,91 milhões de toneladas em 2017 para 10,24 milhões de toneladas em 2017 (FAOSTAT, 2019). A produção asiática é a maior responsável por esse crescimento (Figura 3).

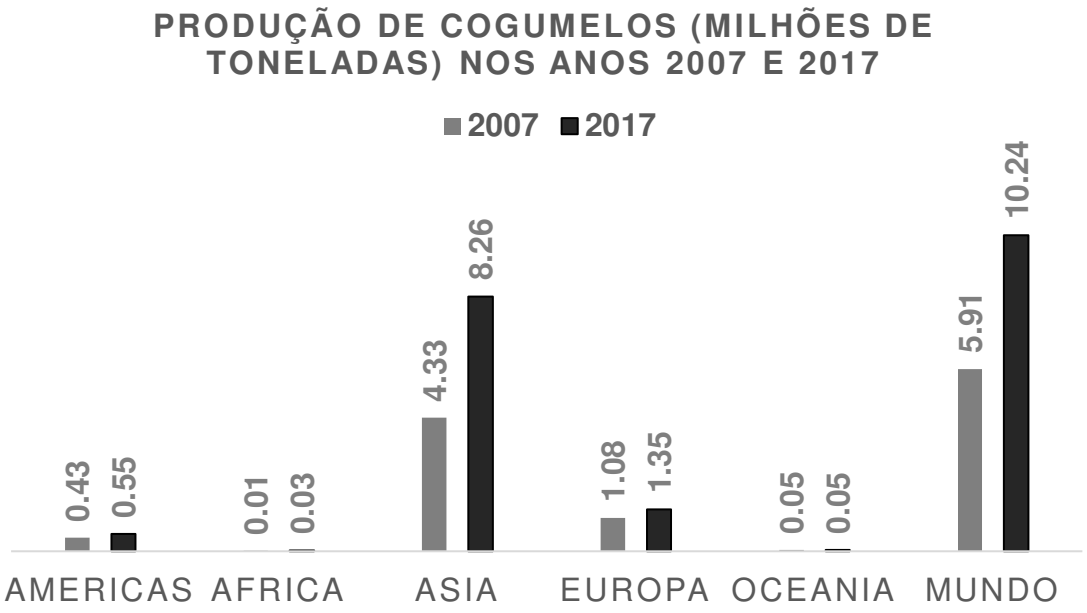

Figura 3. Produção mundial de cogumelos (milhões de toneladas) nos anos 2007 e 2017. Fonte: FAOSTAT, 2019.

No Brasil, as espécies popularmente cultivadas são *Agaricus bisporus*, *Agaricus blazei*, *Lentinula edodes* e algumas espécies do gênero *Pleurotus [Pleurotus ostreatus (shimeji branco e shimeji preto), Pleurotus djamor ou Pleurotus ostreatoroseus, Pleurotus eryngii, Pleurotus pulmonarius e Pleurotus citrinopileatus]* (Furlani & Godoy, 2007; ANPC, 2019). De acordo com a Associação Nacional dos Produtores de Cogumelos (ANPC), estima-se que o Brasil produza pouco mais de 12 mil toneladas de cogumelos *in natura*, sendo a produção do cogumelo Champignon de Paris (*Agaricus bisporus*) a maior, representando 8 mil toneladas por ano. Os cogumelos são comercializados nas formas *in natura*, seco, em conserva, ou em alimentos processados como sopas, molhos e outros.

O consumo de cogumelos no Brasil ainda é pequeno sendo 160 g por pessoa/ano. Esse valor pode ser explicado pela falta de tradição, disponibilidade e desconhecimento da população brasileira em relação aos cogumelos, seus benefícios e, até mesmo, como prepará-los. Em países europeus em que o cogumelo é tradicional na alimentação, como Itália, França e Alemanha, o consumo per capta é superior a 2 kg de cogumelos por pessoa/ano e, em países asiáticos, como China e Coreia do Sul, o consumo de cogumelos é superior a 8 kg de cogumelos por pessoa/ano (ANPC, 2019).

1.2. Valor nutricional e biológico

Cogumelos comestíveis constituem um alimento de elevado valor nutricional, devido à sua composição química. Eles apresentam alta quantidade minerais (principalmente potássio), aminoácidos essenciais, vitaminas (provitamina D_2, vitamina B12) e fibras (Roupas et al., 2012; Kalač, 2013; Feeney et al., 2014). Além disso, os cogumelos são importantes fontes de diferentes tipos de polissacarídeos com propriedades imunomoduladoras (Firenzuoli et al., 2008). Ainda, cogumelos contém diversos fitoquímicos com ampla gama de efeitos na saúde, tais como compostos fenólicos e ácidos orgânicos (Ribeiro et al., 2008). Esses fungos e seus componentes têm sido valorizados pelos diversos benefícios para a saúde, sendo que alguns compostos, frações ou isolados de cogumelos podem impulsionar o sistema imune e ter atividade anticâncer, assim como regular o teor de lipídeos e de glicose no sangue (Firenzuoli et al., 2008; Roupas et al., 2012). Alguns estudos pré-clínicos e clínicos sugerem impactos positivos dos cogumelos na saúde do cérebro e na cognição, controle da obesidade, saúde oral, constipação e diabetes (Roupas et al., 2012; Feeney et al., 2014; Pop et al., 2018).

2. Aminas bioativas

2.1. Definição, classificação e via de síntese das aminas bioativas

As aminas biológicas ativas ou aminas bioativas são base orgânicas de moléculas de pequena massa molecular as quais expressam alta atividade biológica. Elas são formadas através de processos bioquímicos e desempenham diversas atividades biológicas (metabólicas e fisiológicas) importantes nos organismos vivos. As aminas bioativas podem ser encontradas em plantas, animais e fungos (Halász et al., 1994; Silla-Santos, 1996; Gloria, 2005; EFSA, 2011; Fiechter et al., 2013; Papageorgiou et al., 2018).

Como indicado na Figura 4, parte das aminas é denominada de acordo com o respectivo aminoácido precursor, como por exemplo, a histamina, que é originada da histidina, a tiramina da tirosina, a triptamina do triptofano, entre outras. Entretanto, os nomes putrescina e cadaverina estão associados à putrefação e à decomposição, assim como as aminas espermina e espermidina que foram encontradas pela primeira

vez nos fluidos seminais, de onde originaram os nomes (Halász et al., 1994; Glória, 2005; Glória & Vieira, 2007).

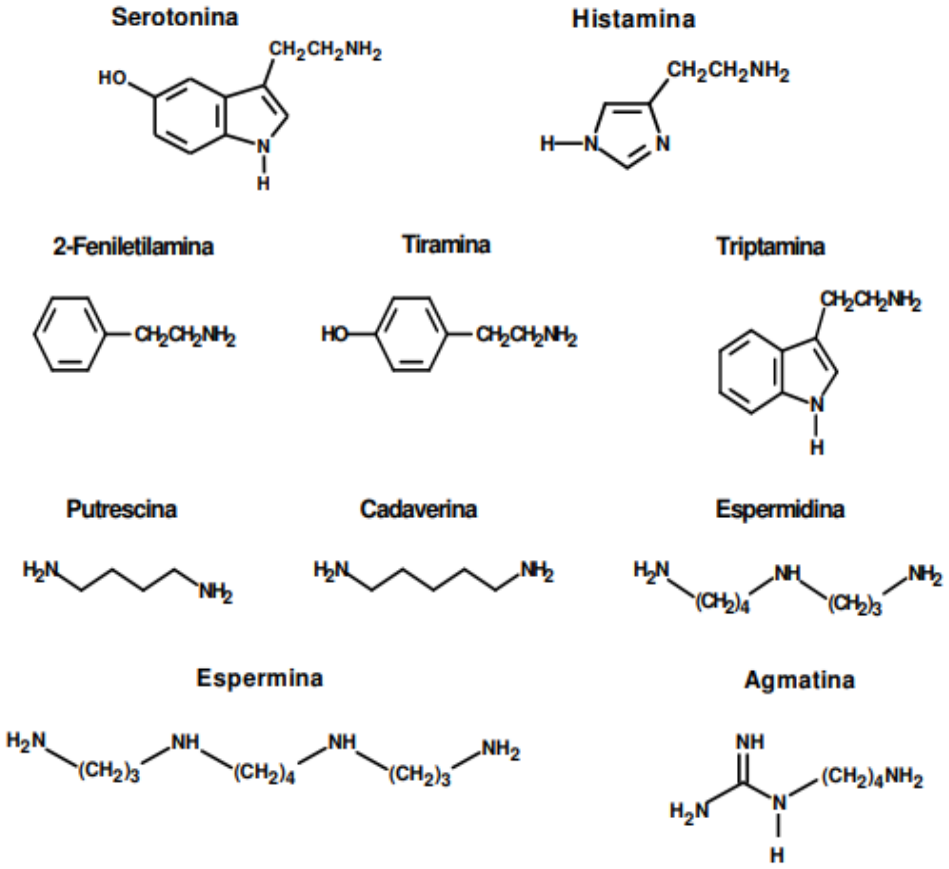

Figura 4. Estrutura química de algumas aminas bioativas (Silva, 2008).

As aminas bioativas podem ser classificadas de acordo com a estrutura química, em alifáticas (putrescina, cadaverina, agmatina, espermina e espermidina) ou aromáticas (tiramina, feniletilamina), ou heterocíclicas (histamina, triptamina e serotonina). Com relação ao número de grupamentos amino, as aminas podem ser classificadas como monoaminas (tiramina e feniletilamina), diaminas (putrescina e cadaverina) ou poliaminas (espermidina e espermina) (Silla-Santos, 1996; Eliassen et al., 2002; Glória 2005).

Quanto à via biossintética, as aminas se classificam em naturais ou biogênicas. As primeiras são formadas durante a biossíntese *in situ*, ou seja, a partir de uma molécula mais simples, à medida que são requeridas (espermina e espermidina) ou podem estar armazenadas nos mastócitos e basófilos (histamina). Por exemplo, a

putrescina é um precursor obrigatório das poliaminas (espermina e espermidina). Além disso, a presença de agmatina em cogumelos sugere uma rota adicional à síntese de poliaminas via arginina, sendo a agmatina formada pela descarboxilação da arginina (Bandeira et al., 2012).

Além da síntese de poliaminas, a putrescina e cadaverina podem contribuir com aroma putrefativo em alimentos. Os limiares relatados na literatura para putrescina e cadaverina são de 10,9 mg/100 g (Wang et al., 1975).

Já as aminas biogênicas podem ser formadas pela descarboxilação de alguns aminoácidos por enzimas descarboxilases microbianas, sendo esta a principal via de formação de aminas nos alimentos (histamina, serotonina, tiramina, feniletilamina, triptamina, putrescina, cadaverina e agmatina) (Kalač & Krízek, 1997; Glória, 2005). Por exemplo, a descarboxilação da histidina forma a histamina, enquanto a tirosina, ornitina, lisina e fenilalanina podem ser descarboxiladas para produzir tiramina, putrescina, cadaverina e feniletilamina, respectivamente (Figura 5) (Redruello, 2017).

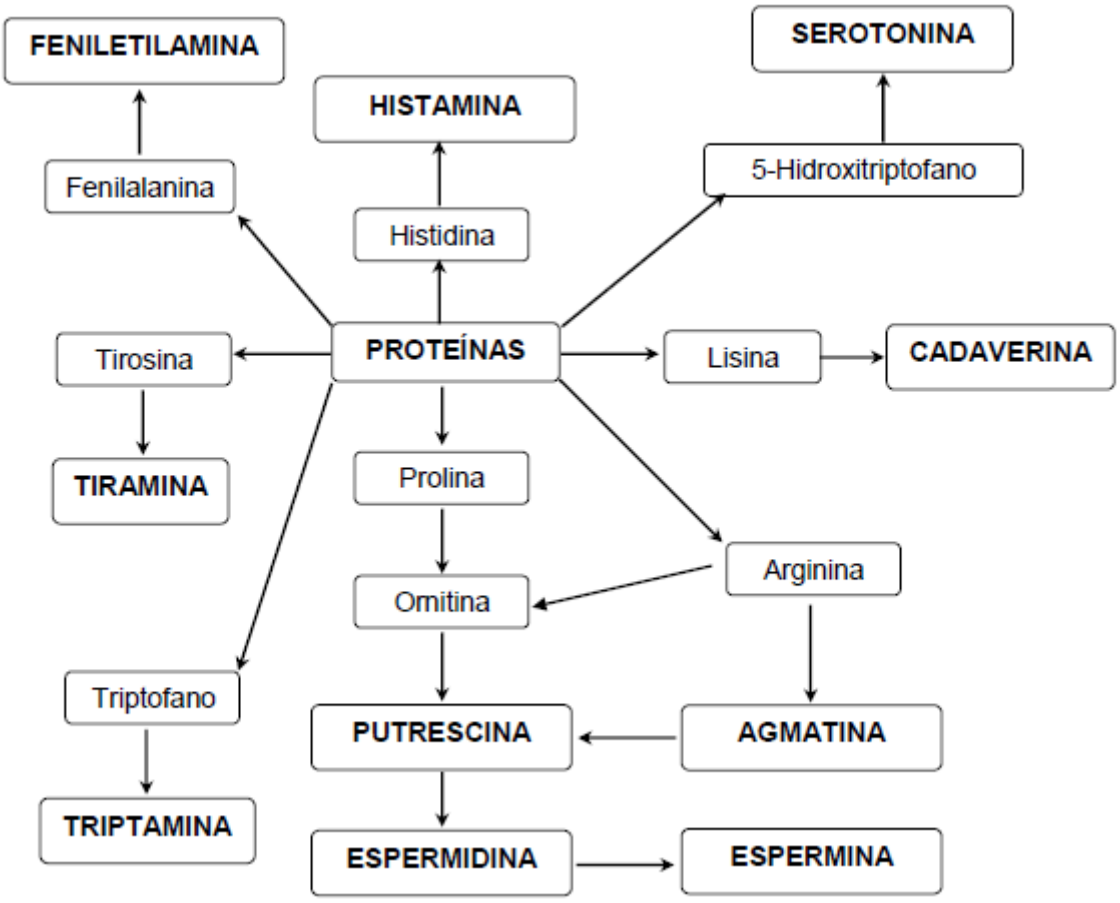

Figura 5. Vias metabólicas para formação de aminas bioativas (Silva, 2008).

2.2. Benefícios e efeitos adversos da ingestão de aminas bioativas

A presença de poliaminas na dieta pode ter diversos efeitos benéficos, incluindo promoção da saúde intestinal, uma vez que as poliaminas são moduladoras da permeabilidade da mucosa intestinal, afetando a absorção de nutrientes e responsáveis pelo crescimento, maturação e regeneração da mucosa (Glória, 2005; Kalač & Krausová, 2005; Kalač, 2014; Ramani et al., 2014; Rogers et al., 2015).

Além disso, as poliaminas podem contribuir para o desenvolvimento do sistema imune, são agentes anti-inflamatórios, apresentando atividade antioxidantes e efeitos cardioprotetores e são efetivas na cicatrização de feridas (Glória, 2005; Kalač, 2014; Ramani et al., 2014; De Cabo & Navas, 2016; Handa et al., 2018; Sharma et al., 2018). A espermina atua, ainda, como reguladora da ativação de macrófagos, linfócitos T e como um inibidor da produção de citocinas pro-inflamatórias (Zahedi et al., 2010).

Nishimura et al. (2006) avaliaram a influência das poliaminas na dieta comparando as mudanças do teor de poliaminas em tecido de ratos. De acordo com os autores, a manutenção das poliaminas nos tecidos biológicos é fundamental para o funcionamento do pâncreas, cérebro e útero no decorrer do envelhecimento dos ratos, e consequentemente, em humanos.

Outro estudo recente, demonstrou que a agmatina tem sido associada com efeitos benefícos à saúde, tais como a neuroproteção no sistema nervoso central, na doença mental, na depressão e na esquizofrenia (Laube & Bernstain, 2017).

Por outro lado, a contribuição das poliaminas para a regeneração celular pode ser considerada indesejada em algumas condições patológicas, uma vez que estão frequentemente presentes em altas concentrações em células com rápida divisão e tecidos em crescimento, sendo associadas ao crescimento tumoral e metástase. É importante observar que as poliaminas não desencadeiam o câncer (Gloria, 2005; Kalač & Krausová, 2005; Kalač, 2014). Entretanto, as poliaminas presentes na dieta de pessoas com câncer podem representar risco à saúde, uma vez que não só estão relacionadas ao crescimento celular, ao aumento do número de células tumorais e do tamanho do tumor, como também com fatores de progressão, metástase e malignidade do câncer (Soda, 2011). Por isso, a indicação nesses casos é a diminuição ou eliminação da ingestão de alimentos que possuam quantidade elevadas de poliaminas, uma vez que elas podem aumentar o crescimento tumoral (Kalač, 2014; Ramani et al., 2014).

Com relação à toxicidade, Del Rio et al. (2018) relataram que a espermidina foi citotóxica nas culturas de células (menor dose para efeito adverso de 1452,50 mg/kg); entretanto, a quantidade de poliaminas ingerida pela dieta estão em concentração distante da concentração tóxica, não representando um problema.

As aminas biogênicas tiramina, triptamina e feniletilamina são aminas neuro e vasoativas que, se presentes em elevadas concentrações nos alimentos, estão associadas a crises hipertensivas e quadros de enxaquecas. A sensibilidade aos efeitos tóxicos está diretamente relacionada com a biodisponibilidade destas aminas. Pessoas que fazem o uso de drogas da classe dos inibidores da monoamina oxidase (IMAOs) são mais sensíveis à intoxicação. O álcool também é considerado como potencializador dos efeitos tóxicos das aminas biogênicas por ser antagonista das enzimas monoamina oxidases. Além disso, a presença das aminas putrescina e cadaverina potencializa a toxicidade dessas aminas por apresentarem relação competidora com as demais aminas biogênicas na reação com as enzimas aminoxidases (EFSA, 2011).

2.3. Aminas bioativas em cogumelos

Além dos componentes relatados, os cogumelos podem ser fonte de aminas bioativas, dentre elas, agmatina, cadaverina, espermina, espermidina, feniletilamina, putrescina, tiramina e triptamina, sendo a espermidina a amina com maiores teores em cogumelos (Yamoto et al.,1982; Okamoto et al., 1997; Nishimura et al., 2006; Nishibori et al., 2007; Dadaková et al., 2009; Reis, 2014).

2.3.1. Ocorrência de aminas bioativas em cogumelos

Afora as aminas que ocorrem naturalmente, cogumelos são susceptíveis a formação e ao acúmulo de aminas biogênicas devido a elevada atividade de água e a presença de aminoácidos livres (Tsai et al., 2007; Sun et al., 2017; Manninen et al., 2018; Rotola-Pukkila, 2019), que são condições favoráveis para o crescimento microbiano e consequente deterioração dos cogumelos (Dadáková et al., 2009; EFSA, 2011; Kalač, 2013; Feeneey et al., 2014; Papageorgiou et al., 2018).

Os estudos que investigaram o perfil e teor de aminas em cogumelos estão apresentados na Tabela 1. Essas aminas foram estudas incialmente por Yamamoto

et al. (1982) no intuito de avaliar o potencial dos alimentos em formar nitrosaminas. Esses autores desenvolveram método analítico para determinação das aminas espermidina, espermina, putrescina e cadaverina em diversos alimentos, dentre eles seis espécies de cogumelos (*Agaricus bisporus*, *Collybia veltipes*, *Pholiota nameko*, *Pleurotus ostreatus*, *Lentinus edodes* e *Auricularia polytricha*). Destaca-se nesse estudo a presença de espermidina e putrescina em todos os cogumelos analisados e o elevado teor de espermidina (Kalač, 2014) nos cogumelos frescos variando de 8,0 (*Lentinus edodes*) a 21,3 mg/100 g (*Pleurotus ostreatus*).

Kalač & Křížec (1997) avaliaram a ocorrência das aminas putrescina, cadaverina, histamina e tiramina em cinco espécies de cogumelos (*Boletus badius, Boletus chysentereon, Boletus variegatus* e *Agaricus bisporus*). Histamina e tiramina não foram detectadas. Os valores de cadaverina foram baixos, variando de não detectado a 0,94 (*Boletus badius*) e o teor de de putrescina variou de não detectado a 8,04 mg/100 g.

Okamoto et al. (1997) determinaram o teor de agmatina, cadaverina, histamina, putrescina, espermidina, espermidina em diversos alimentos, entre eles os cogumelos *Lentinus edodes*, *Lyophyllum shimeji* e *Flammulina velutipes*. A agmatina foi identificada apenas no cogumelo *Lyophyllum shimeji*. A espermidina foi a amina com maior teor sendo identificada em todos os cogumelos variando de 6,97 (*Lyophyllum shimeji*) a 12,93 mg/100 g (base úmida) (*Lentinus edodes*). A espermina foi quantificada em maior quantidade (1,38 mg/100 g base úmida) no cogumelo *Flammulina velutipes* e a putrescina foi identificada em todos os cogumelos sendo maior no cogumelo *Lyophyllum shimeji* (1,85 mg/100 g base úmida). Com relação a histamina e cadaverina, esses autores falharam em separar e essas duas aminas que foram quantificadas juntas nos cogumelos *Lentinus edodes*.

Nishimura et al. (2006) avaliaram os cogumelos *Lyophyllum shimeji, Pleurotus eryngii, Grifola frondosa, Lentinus edodes, Pholiota nemeko, Hericium erinaceum, Agaricus blazei murrill, Tricholoma matsutake* e *Flammulina velupite.* A espermidina e a cadaverina foram detectadas em todos os cogumelos e o teor da primeira variou de 5,80 (*Tricholoma matsutake*) a 15,83 mg/100 g (base úmida) (*Lyophyllum shimeji*) e foi quantificada em 33,99 mg/100 g (base seca) em *Agaricus blazei.* O teor de cadaverina variou de 0,59 (*Grifola frondosa*) a 5,72 mg/100 g (base úmida) (*Hericium erinaceu*). Apenas na espécie *Pleurotus eryngii* não foi detectada a putrescina. Nos cogumelos em que foi detectada, a putrescina variou de 0,30 *(Flammulina velupite*) a

9,08 mg/100 g (base úmida) (*Tricholoma matsutake*). A espermina somente foi detectada no cogumelo *Agaricus bisporus* seco, apresentando o teor de 0,34 mg/100 g (base seca).

Nishibori et al. (2007) avaliaram a ingestão de poliaminas através dieta no Japão. Foi determinado o teor de putrescina, espermidina e espermina em diversos alimentos, entre eles os cogumelos *Flammulina velutipes*, *Lentinus edodes* e *Lyophyllum shimeji*, além de um cogumelo que eles denominaram pela palavra "*mushroom*". Em todos os cogumelos analisados foram encontradas espermidina e putrescina. A espermidina foi a poliamina que apresentou maior concentração variando de 2,22 (*Lyophyllum shimeji*) a 8,86 mg/100 g (base úmida) ("*mushroom*") e a putrescina variou de 0,02 (*Lentinus edodes*) a 0,95 mg/100 g (base úmida) (*Flammulina velutipes*). A espermina foi identificada apenas nas amostras *Lyophyllum shimeji* (0,04 mg/100 g, base úmida) e "mushroom" (0,34 mg/100 g, base úmida).

Dadaková et al. (2009) analisaram pela primeira vez diversos cogumelos selvagens colhidos na região South Bohemia na República Tcheca. Foram analisados os teores das aminas espermidina, espermina, putrescina, feniletilamina, triptamina e tiramina. A espermidina foi a única amina presente em todos os cogumelos; seu teor variou de 0,61 mg/100 g (base úmida) (*Sparassis crispa*) a 38,43 mg/100 g (base úmida) (*Boletus erythropus*). A putrescina foi detectada na maioria das espécies com exceção das espécies *Sparassis crispa* e *Coprinus comatus*, sendo que a espécie *Boletus erythropus* apresentou o maior teor (18,58 mg/100 g, base úmida). O teor máximo de feniletilamina foi encontrado na espécie *Xerocomus* (3,65 mg/100 g, base úmida). Triptamina e tiramina foram detectadas em poucas espécies, sendo a espécie *Xerocomus badius* que apresentou o maior teor dessas aminas (0,67 mg/ 100 g, para triptamina e 0,72 mg/100 g - base úmida, para tiramina). A espermina apresentou-se na grande maioria das amostras em valores pouco expressivos.

Reis (2014) investigou o teor de aminas bioativas (espermidina, agmatina, putrescina, cadaverina, feniletilamina, triptamina, tiramina, histamina e serotonina) em oito cogumelos das variedades Champignon e Portobello da espécie *Agaricus bisporus*, Shitake da espécie *Lentinula edodes*, Shimeji preto, Shimeji branco, Hiratake, Salmão e Eryngii do gênero *Pleurotus*. Nesse estudo, pela primeira vez, diferentes tipos de cogumelos foram agrupados com base no perfil e teor de aminas bioativas. De nove aminas analisadas, apenas a histamina e serotonina não foram detectadas em nenhum cogumelo. A espermidina foi detectada em todos os

cogumelos e seu teor variou de 6,26 mg/100 g (base úmida) na espécie *Lentinula edodes* a 12,4 mg/100 g (base úmida) na variedade Shimeji preto (*Pleutorus sp.*). A agmatina foi encontrada na maioria das variedades estudadas ausentando-se apenas nas variedades da espécie *A. bisporus*. Essa amina foi inerente ao gênero *Pleurotus*, sendo detectada em grande quantidade na espécie salmão 20,9 mg/100 g (base úmida). A triptamina foi determinada em 6 variedades de cogumelos apresentando teor máximo de 1,37 mg/100 g (base úmida) na variedade Shimeji branco. Feniletilamina, tiramina e putrescina foram encontradas em apenas 4 variedades de cogumelos e apresentaram valor máximo para feniletilamina de 0,84 mg/100 g (base úmida) na variedade Shimeji branco, 0,88 mg/100 g (base úmida) para tiramina, 2,48 mg/100 g (base úmida) para putrescina na variedade Salmão. A cadaverina foi detectada em apenas três espécies apresentando maior valor de 0,59 mg/100 g (base úmida) na variedade Salmão. De acordo com o autor, é possível afirmar que a espermidina é inerente a todos os cogumelos e a agmatina é inerente aos cogumelos Shitake, Shimeji Branco, Shimeji Preto, Salmão, Hiratake e Eryngii. Todos os cogumelos analisados podem ser utilizados na dieta como fonte de espermidina. Os altos teores de agmatina encontrados nos cogumelos Salmão, Hiratake, Shimeji Branco e Shimeji Preto, podem indicar que a síntese de poliaminas nesses tipos de cogumelos pode ocorrer também via agmatina. Nos cogumelos nos quais foram detectadas as aminas vasoconstritoras tiramina, triptamina e feniletilamina, essas aminas estavam presentes em quantidades pequenas, insuficientes para causar efeitos adversos a saúde. Os teores das aminas cadaverina, putrescina, tiramina, triptamina e feniletilamina foram discretos com relação aos teores totais.

Apesar dos poucos estudos que investigaram o perfil e teor de aminas em cogumelos é possível perceber que os cogumelos apresentam elevado teor de espermidina e essa amina é inerente a este alimento. Baseado na classificação proposta por Kalač (2014), com relação aos teores de poliaminas pelos estudos analisados, os cogumelos analisados podem ser considerados alimentos com teor elevado (> 1 mg/100 g) ou muito elevado (>10 mg/100 g) em poliaminas.

A diferença na ocorrência de aminas entre espécies de cogumelos pode estar associada à genética, substrato utilizado na produção de cogumelos, tempo de colheita, estágio de maturação, tratamento pós-colheita e fatores abióticos ambientais (Glória, 2006; Jiaojiao et al., 2018; Meng et al., 2019). Além desses fatores, carece na literatura dados sobre a influência do processamento, bioacessibilidade das aminas

bioativas nos cogumelos e possível influência desse alimento na formação de aminas bioativas no intestino.

2.3.2. Influência do processamento e do armazenamento no teor de aminas bioativas em cogumelos

O uso de aminas bioativas como índice de qualidade em cogumelos foi estudado por Yen (1992) e Kalač & Krizek (1997). Yen (1992) avaliou o teor das aminas bioativas cadaverina, histamina, feniletilamina, putrescina, triptamina e tiramina como indicador de qualidade no cogumelo *Volvariella volvacea* (cogumelo palha) nas formas fresco, cozido e enlatado. Foi observada uma redução de 80% no teor total de aminas, passando de 14,77 mg/100 g para 2,81 mg/100 g (base úmida). Esta perda foi atribuída à lixiviação das aminas para a água de cozimento, uma vez que a histamina, a putrescina e tramina são termoestáveis. Os teores de aminas nos cogumelos enlatados foram menores do que os valores encontrados para os cogumelos cozidos. As concentrações de aminas biogênicas no cogumelo palha foram reduzidas com o tratamento térmico. Foi avaliado também o efeito do armazenamento a 4 e 25 °C por um período de cinco dias sobre os teores de aminas nos cogumelos. O teor das aminas aumentou com o tempo de armazenamento a 4 °C e 25 °C, sendo o aumento maior a 25 °C do que a 4 °C. Este resultado demonstra que quanto maior a temperatura maior a velocidade de deterioração.

Em trabalho realizado por Kalač & Křížec (1997), em cinco espécies de cogumelos (*Boletus badius, Boletus chysentereon, Boletus variegatus* e *Agaricus bisporus*) foram estudadas as alterações nos teores das aminas putrescina, cadaverina, histamina e tiramina de acordo com o processamento e diferentes temperaturas e tempos de estocagem. Observou-se que após armazenamento a 6 C e a 20°C por 48 horas, os teores de cadaverina e de putrescina aumentaram em todas as espécies. No *Agaricus bisporus*, por exemplo, esses teores não eram detectáveis no cogumelo recém colhido e, após o armazenamento a 20 °C por 48 horas, estes teores passaram para 36,8 mg/100 g para putrescina e 3,69 mg/100 g para cadaverina (base seca).

Tabela 1. Perfil e teor de aminas bioativas em cogumelos comestíveis.

Espécie	Amostras (n)	Faixa de concentração de aminas bioativas (mg/100 g)										Referência
		AGM	CAD	HIM	FEM	PUT	SRT	TRP	TIM	EPD	EPM	
Agaricus bisporus	2		nd			0,09-0,22				16,29-19,22	0,21-0,30	Yamoto et al., 1982
Collybia veltipes	2		0,10-0,52			0,59-0,63				9,33-9,74	nd	
Pholiota nameko	1		0,81			2,44				8,56	0,33	
Pleurotus ostreaus	2		0,06-0,25			0,21-1,28				17,63-21,34	0,07-0,10	
Lentlnus edodes	2		0,15-0,20			0,14-0,19				8,00-8,38	nd	
Volvariella volvacea	Não informado		nd-47,24	nd-24,44	0,33-61,32	0,05-48,09		nd-14,17	0,33-43,12			Yen, 1992
Boletus badius	Não informado		nd-16,5			4,68-163						Kalac & Krizek, 1997
Boletus chrysentereon			nd-20,0			4,96-505						
Boletus variegaus			nd-13,3			4,32-62,5						
Agaricus bisporus			nd-3,69			nd-36,8						
Lentinus edodes	2	nd	<0,05	<0,06		0,26				12,93	nd	Okamoto et al., 1997
Lyophyllum shimeji	2	<0,07	nd	nd		1,85				6,97	1,38	
Flammulina velutipes	2	nd	<0,10	nd		<0,13				8,72	<0,10	
Lyophyllum shimeji	2	nd	1,06			1,08-4,53				5,75-15,83	nd	Nishimura et al., 2006
Pleurotus eryngii	2	7,68	2,16			nd				15,69	nd	
Grifola Frondosa	2	nd	0,59			1,18				10,57	nd	
Lentinus edodes	2	nd	0,78			0,09				10,07	nd	
Pholiota nameko	2	nd	0,31			0,37-1,45				8,83	nd	
Hericium erinaceu	2	nd	1,32-5,72			1,23				7,16	nd	

nd = não detectado.

Tabela 1. Perfil e teor de aminas bioativas em cogumelos comestíveis (continuação).

Espécie	Amostras (n)	Faixa de concentração de aminas bioativas (mg/100 g)										Referência
		AGM	CAD	HIM	FEM	PUT	SRT	TRP	TIM	EPD	EPM	
Agaricus blazei	2	nd	5,27			5,95				33,99	0,34	Nishimura et al., 2006
Tricholoma masutake	2	nd	0,68			9,08				5,80	nd	
Flammulina velutipes	2	nd	1,61			0,30				8,05	nd	
Flammulina velutipes	6					0,44				3,25	nd	Nishibori et al., 2007
"mushroom"	5					0,40				8,86	0,34	
Lentinus edodes	6					0,02				5,64	nd	
Lyophyllum shimeji	5					0,95				2,22	0,04	
Xerocomus badius	18				1,30-1,84	8,41-11,33		0,66-0,67	nd-0,72	5,39-8,43	nd-0,39	Dadaková et al., 2009
Xerocomus chrysentereon	22				3,56-3,78	12,24-15,56		nd-0,43	nd-0,28	0,91-12,04	nd-0,25	
Suillus variegaus	17				nd-0,26	6,68-9,25		nd-0,22	nd-0,22	2,42-3,70	0,28-0,52	
Suillus grevillea	13				nd	3,23-5,14		nd	nd	1,98-3,42	0,22-0,36	
Suillus tridentinus	6				nd	4,12		nd	nd	1,92	0,24	
Cantharellus cibarius	5				nd	0,71		nd	nd	1,42	0,46	
Cantharellus tubaeformis	60				0,27	1,22		nd	nd	2,52	1,26	
Lactaarius deterrimus	8				0,53	0,27		nd	0,14	0,51	0,17	
Lactaarius pinicola	8				0,62	0,27		nd	0,21	2,32	1,17	
Amanita Rubescens	6				0,23	0,38		nd	0,27	7,08	nd	

nd = não detectado.

Tabela 1. Perfil e teor de aminas bioativas em cogumelos comestíveis (continuação).

Espécie	Amostras (n)	Faixa de concentração de aminas bioativas (mg/100 g)										Referência
		AGM	CAD	HIM	FEM	PUT	SRT	TRP	TIM	EPD	EPM	
Armillaria mellea	45				0,35	0,24		nd	0,24	3,78	0,59	Dadaková et al., 2009
Coprinus comaus	7				0,13	Nd		nd	nd	0,69	8,03	
Boletos edulis	4				0,48-1,81	9,81-10,69		nd	nd	1,26-3,55	0,22-0,61	
Boletus erythropus	2				0,46	18,58		nd	nd	38,43	nd	
Xerocomus subtomentosus	3				3,84	8,49		nd	nd	1,05	nd	
Lepiota procera	3				nd	0,70		nd	0,23	12,31	0,43	
Sparassis crispa	1				nd	nd		0,47	nd	0,61	0,82	
Agaricus bisporus	10	nd	nd	nd	nd	nd	nd	nd-0,13	nd-0,01	9,72-9,83		Reis, 2014
Lentinula edodes	5	0,49	nd	nd	0,05	nd	nd	0,02	nd	6,26		
Pleurotus ostreatus	25	2,36-20,90	nd-0,59	nd	nd-0,84	nd-2,48	nd	nd-1,37	nd-0,88	7,77-12,4		

nd = não detectado. AGM = agmatina, CAD = cadaverina, HIM = histamina, FEM = feniletilamina, PUT = putrescina, SRT = serotonina, TRP = triptamina, TIM = tiramina, EPD = espermidina, EPM = espermina.

Quando foram avaliados os cogumelos fatiados estocados a 6 °C, houve um aumento no teor de putrescina de 62,9 mg/100 g no dia 2 para 163,0 mg/100 g no dia 7 para o cogumelo *Boletus chrysentereon* e para a cadaverina este aumento foi de 1,14 mg/100 g no dia 2 para 200 mg/100 g no dia 7. Na temperatura de estocagem a 20°C, após 48 horas, os cogumelos já estavam estragados e com teores de putrescina e cadaverina elevados (505 mg/100 g e 102 mg/100 g, respectivamente). Diante disso, é sugerido o uso dessas duas aminas como índice de qualidade para cogumelos.

2.3.3. Métodos analíticos para determinação de aminas bioativas em cogumelos

São diversas as razões para determinar aminas bioativas em cogumelos, dentre elas, a presença de aminas bioativas são indicadoras do potencial biológico, da qualidade e do efeito adverso (Křížec, 1991; Kalač, 2014). Dessa forma torna-se necessário o desenvolvimento de métodos confiáveis, sensíveis e rápidos para determinação das aminas bioativas em cogumelos.

Devido à propriedade química das aminas bioativas, a determinação dessas substâncias em alimentos é desafiadora e trabalhosa (Křížec, 1991; Önal et al., 2013; Papageorgiou et al., 2018). As principais características que tornam difícil a determinação de aminas bioativas em alimentos são a ausência de um cromóforo e a difícil separação em coluna de fase reversa.

Diversas técnicas têm sido relatadas para análise de aminas bioativas em alimentos, dentre elas, a cromatografia líquida de alta e ultra eficiência (HPLC/UHPLC), a cromatografia gasosa (GC) e a eletroforese capilar (CE) (Önal et al., 2013). Além desses métodos, outras abordagens não cromatográficas para determinação de aminas bioativas em alimentos foram desenvolvidas como métodos imuno-enzimáticos, kits para determinação de histaminas semi-quantitativo, biosensores e amperométricos (Papageorgiou et al., 2018). Os métodos utilizados para preparo de amostra e detecção e quantificação de aminas bioativas em cogumelos estão demonstrados nas Tabelas 2 e 3, respectivamente.

Em geral, as aminas bioativas são extraídas dos cogumelos mediante a extração ácida com ácido perclórico ($HClO_4$) (Yamoto et al., 1982; Yen, 1992; Kalač & Křížec, 1997) ou ácido tricloroacético (TCA) (Okamoto et al., 1997; Nishimura et al., 2006; Reis, 2014) seguido de agitação, centrifugação e separação do sobrenadante por filtração simples.

Tabela 2. Métodos de preparo da amostra para determinação de aminas bioativas em cogumelos.

Aminas bioativas	Preparo da amostra	Referência
CAD, EPD, EPM PUT	Cogumelo foi homogeneizado com $HClO_4$ a 2% agitado e mantido a 4˚C durante a noite. A suspensão foi centrifugada e o precipitado foi lavado duas vezes com $HClO_4$ a 2%, centrifugado novamente, filtrado e aplicado na coluna de resina de troca catiônica (9 mm id; 3 mL de volume). A coluna foi lavada sucessivamente com tampão de fosfato 0,1 mol/L contendo NaCl a 0,1 M e HCl 1 mol/L. O eluato foi colhido e adicionado padrão interno. A mistura foi evaporada até em evaporador rotativo a 50 °C sob vácuo. O resíduo foi recuperado com adição de NaOH a 10% e cloroformato de etilo. A mistura foi agitada e os derivatizados resultantes foram extraídos 3 vezes com de éter dietílico. Os extratos combinados foram evaporados até à secura a 50 ° C com uma corrente suave de nitrogênio. O resíduo foi dissolvido de acetato de etilo e adicionou-se alguns grãos de Na_2SO_4 anidro.	Yamoto et al., 1982
CAD, FEM, HIM, PUT, TIM, TRP	O cogumelo foi homogeneizado por $HClO_4$ a 6%. A mistura foi armazenada a 4˚C durante a noite e filtrada. O filtrado foi ajustado para pH 6,5 com KOH, depois armazenado a 4 ˚C durante 2 h e filtrado. O filtrado foi derivatizado com cloreto de dansila.	Yen, 1992
PUT, CAD	Cogumelo foi homogeneizado com $HClO_4$ e agitado.	Kalač & Křížec, 1997
AGM, CAD, EPD, EPM, HIM, PUT	Cada cogumelo foi homogeneizado em ácido tricloroacético 5% e depois centrifugado. Uma alíquota do sobrenadante foi aplicada a uma coluna de resinas de troca iônica (Muromac CR-70) para eliminar os aminoácidos e peptídeos que interferem nas análises. As poliaminas foram eluídas com HCl 6 mol/L, que foi subsequentemente removido por evaporação.	Okamoto et al., 1997
AGM, CAD, EPD, EPM, PUT	Os cogumelos foram congelados em nitrogênio líquido, cortados em pequenos pedaços e homogeneizados com ácido tricloroacético a 5%. Em seguida foi centrifugado e o sobrenadante foi purificado em duas colunas de troca iônica TSK gel (4,6 x 50 mm) para separar as poliaminas dos aminoácidos.	Nishimura et al., 2006
EPD, EPM, PUT	As amostras foram homogeneizadas em ácido tricloroacético a 5% e armazenadas a -20˚C até a análise. As amostras foram centrifugadas sobrenadantes contendo poliaminas livres foram analisados.	Nishibori et al., 2007
EPD, EPM, FEM, PUT, TIM, TRP	Os cogumelos em pó liofilizados foram homogeneizados com H_2ClO_4 a 0,6 mol/L. A mistura foi agitada e centrifugada. O sobrenadante foi filtrado e as aminas extraídas foram derivatizadas com cloreto de dansila.	Dadaková et al., 2009
AGM, CAD, EPM, FEM, HIM, PUT, SRT, TIM, TRP	Cogumelos frescos foram homogeneizados com ácido tricloroacético. As amostras foram agitadas e centrifugadas. O sobrenadante foi filtrado.	Reis, 2014

AGM = agmatina, CAD = cadaverina, HIM = histamina, FEM = feniletilamina, PUT = putrescina, SRT = serotonina, TRP = triptamina, TIM = tiramina, EPD = espermidina, EPM = espermina.

Para a purificação dos extratos, alguns autores usaram colunas de troca iônica para eliminar interferentes como aminoácidos e peptídeos (Yamoto et al., 1982; Okamoto et al., 1997; Nishimura et al., 2006).

As técnicas de separação utilizadas foram a cromatografia gasosa (Yamoto et al., 1982), a cromatografia líquida com derivatização pré-coluna (Yen,1992; Kalač & Křížec, 1997; Dadakova et al., 2009) e derivatização pós-coluna (Okamoto et al., 1997; Nishimura et al., 2006; Nishibori et al., 2007; Reis, 2014). O método por cromatografia gasosa (Yamoto et al., 1982) depende de preparo de amostra extenso e trabalhoso, com inúmeras etapas, incluindo hidrólise ácida, purificação com coluna de troca iônica para separação das aminas bioativas de aminoácidos e peptídeos e derivatização no intuito de permitir o arraste das aminas pela coluna. A separação foi feita em coluna empacotada, o que dificulta a reprodutibilidade do método. A identificação foi realizada mediante a detecção por espectrometria de massas e quantificação através da detecção por ionização de chamas (FID).

Com avanço da cromatografia líquida de alta eficiência, essa técnica foi sendo preferida para determinação de aminas bioativas em alimentos e, portanto, nos cogumelos (Önal et al., 2013; Papageorgiou et al., 2018). Pela estrutura química (Figura 4), as aminas bioativas apresentam elevada polaridade e, assim, são separadas com melhor eficiência em colunas de troca iônica, comparada a coluna C18. Dessa forma, a derivatização pré-coluna tem sido utilizada por diversos autores (Okamoto et al., 1997; Nishimura et al., 2006; Nishibori et al., 2007; Reis, 2014) para determinação de aminas bioativas em alimentos a fim de alterar as propriedades físico químicas desses compostos e oferecer um grupo hidrofóbico, aumentando a interação do derivatizado com a fase estacionária. O derivante cloreto de dansila tem sido o reagente mais usado para derivatização de aminas. Os derivatizados de dansila podem ser separados tanto por fase reversa quanto por fase polar. A fase reversa é mais usada por apresentar melhor resolução na separação. Os derivatizados de dansila podem ser detectados por detectores ultravioleta ou por de fluorescência (Yen, 1992; Dadaková et al., 2009).

Tabela 3. Métodos de detecção e quantificação de aminas bioativas em cogumelos.

Aminas bioativas	Sistema/Fase estacionária	Derivatização/ Detecção	Tempo de corrida	Referências
CAD, EPD, EPM PUT	Cromatógrafo gasoso modelo Shimadzu 4CM com detector de ionização por chamas Cromatógrafo gasoso modelo Shimadzu LKB 9000 com detector por espectrometria de massas Coluna de vidro (3,0 x 500 mm)	Ionização por chamas (FID) Espectrometria de massas (MS)	20 min	Yamoto et al., 1982
CAD, FEM, HIM, PUT, TIM, TRP	HPLC Hitachi com detector UV-Vis	Derivatização pré-coluna com cloreto de dansila Detecção a 254 nm	Não informado	Yen, 1992
PUT, CAD	HPLC detector UV/vis Coluna C18 modelo SGX-C18 (3,0 x 150 mm, 5µm)	Derivatização com cloreto de benzoíla Detecção a 254 nm	Não informado	Kalač & Křížec, 1997
AGM, CAD, EPD, EPM, HIM, PUT	HPLC Coluna de troca ionica modelo TSK-gel Aminopak column	Detecção pós coluna por fluorescência por reação com ortoftaladeído	Não informado	Okamoto et al., 1997
AGM, CAD, EPD, EPM, PUT	HPLC Coluna de troca iônica modelo TSK- gel IEX215 (4,0 x 80 mm)	Detecção pós coluna por fluorescência por reação com ortoftaladeído excitação a 340 nm e emissão a 455 nm	102 min	Nishimura et al., 2006
EPD, EPM, PUT	HPLC Hitachi modelo 2619F Coluna com resina de de troca cationica (4,0 x 50 mm)	Detecção pós coluna com ortoftaladeído Fluorescência	Não informado	Nishibori et al., 2007
EPD, EPM, FEM, PUT, TIM, TRP	HPLC modelo RR-LC Agilent Coluna C18 Zorbax Eclipse XDB-C18 (4,6 x 50 mm, 1,8 µm).	Derivatização pré-coluna com cloreto de dansila Detecção a 225 nm	12 min	Dadaková et al., 2009
AGM, CAD, EPM, FEM, HIM, PUT, SRT, TIM, TRP	HPLC modelo Shimadzu LC-10AD acoplado com espectrofluorímetro Coluna C18 Novapack (3,6 x 300 mm) (Waters)	Detecção pós coluna com ortoftaladeído Fluorescência excitação a 340 nm e emissão a 455 nm	60 min	Reis, 2014

AGM = agmatina, CAD = cadaverina, HIM = histamina, FEM = feniletilamina, PUT = putrescina, SRT = serotonina, TRP = triptamina, TIM = tiramina, EPD = espermidina, EPM = espermina.

O cloreto de benzoíla também pode ser usado como derivante (Křížec, 1991; Kalač & Křížec, 1997) e traz algumas vantagens, por ser um derivante barato, de fácil acesso e a duração do derivatizado é menos crítica que o derivatizado cloreto de dansila. O processo de reação acontece à temperatura ambiente e não há necessidade de tampão. Em comparação com dansilaminas, benzamidas não são sensíveis a luz e podem ser estocadas a -20 ˚C por diversos meses (Křížec, 1991).

Na técnica de derivatização pós-coluna, o reagente orto-ftalaldeído foi utilizado como derivante (Okamoto et al., 1997; Nishimura et al., 2006; Nishibori et al., 2007) ou par-iônico (Reis, 2014). A separação cromatográfica por par-iônico permite o uso de coluna de fase reversa e oferece seletividade adicional ao permitir manipular a concentração do reagente que forma o par iônico. A eficiência da separação se dá pela restauração do par iônico ao longo dos gradientes na corrida (Collins et al., 2007). A técnica de troca iônica consiste em uma fase estacionária com uma matriz na qual são ligados covalentemente grupos iônicos, cuja carga é neutralizada pelo contra-íon. A eficiência da separação se dá pela capacidade de troca iônica e a seletividade e retenção na troca iônica é fortemente influenciada pelo tamanho da carga dos analitos, tipo de trocado iônico, temperatura da coluna e diversas características da fase móvel, tais como pH, força iônica, vazão, modificador orgânico, contra-íon, concentração do contra-íon e tipo de solução tampão (Collins et al., 2007). Ambas as técnicas cromatográficas (troca iônica e par-iônico), permitem o preparo de amostra mais simples, reduz o efeito matriz e o viés referente a influência do analista no preparo de amostra, apresentando assim boa reprodutibilidade. Contudo essas técnicas apresentam um tempo elevado da corrida cromatográfica e consumo elevado do derivante quando comparado a técnica de derivatização pré-coluna (Okamoto et al., 1997; Nishimura et al., 2006; Nishibori et al., 2007).

3. Aminoácidos em cogumelos

Além de serem precursores das aminas bioativas (Figura 5), os aminoácidos são componentes essenciais na dieta humana. Por definição, os aminoácidos são compostos orgânicos formados por um grupo amino (—NH3) associado a um grupo carboxila (—COOH). São 20 os diferentes tipos de aminoácidos mais encontrados em alimentos (Figura 6), dos quais nove são aminoácidos essenciais (isoleucina, leucina, lisina, metionina, fenilalanina, treonina, triptofano, valina e histidina) e 11 não

essenciais (alanina, arginina, asparagina, ácido aspártico, cisteína, ácido glutâmico, glutamina, glicina, prolina, serina e tirosina). A principal função dos aminoácidos é atuar como subunidades de estruturação de proteínas. Eles são indispensáveis para as funções vitais, uma vez que estão envolvidos na biossíntese de enzimas, hormônios e proteínas estruturais (Arrieta & Prats-Moya, 2012).

Além disso, existem evidências dos benefícios da suplementação com aminoácidos em quadros clínicos como disfunção metabólica, reabsorção insuficiente, aumento da demanda nutricional após o trauma cirúrgico e atendimento médico de recém-nascidos (Marchini et al., 2016).

Outro benefício da presença de aminoácidos livres nos alimentos é a sua contribuição para o sabor de diversos alimentos (Mau et al., 2001; Yang et al., 2001). O sabor representa uma das qualidades mais importantes que contribuem para o grande consumo de cogumelos cultivados. O sabor típico dos cogumelos está relacionado à presença de pequenos compostos voláteis e não-voláteis, como as substâncias solúveis em água, incluindo 5'-nucleótidos livres, aminoácidos livres e carboidratos solúveis. Os aminoácidos que mais contribuem para o gosto de cogumelos são a alanina, os ácidos aspártico e glutâmico, a glicina, e a treonina (Tseng & Mau, 1999).

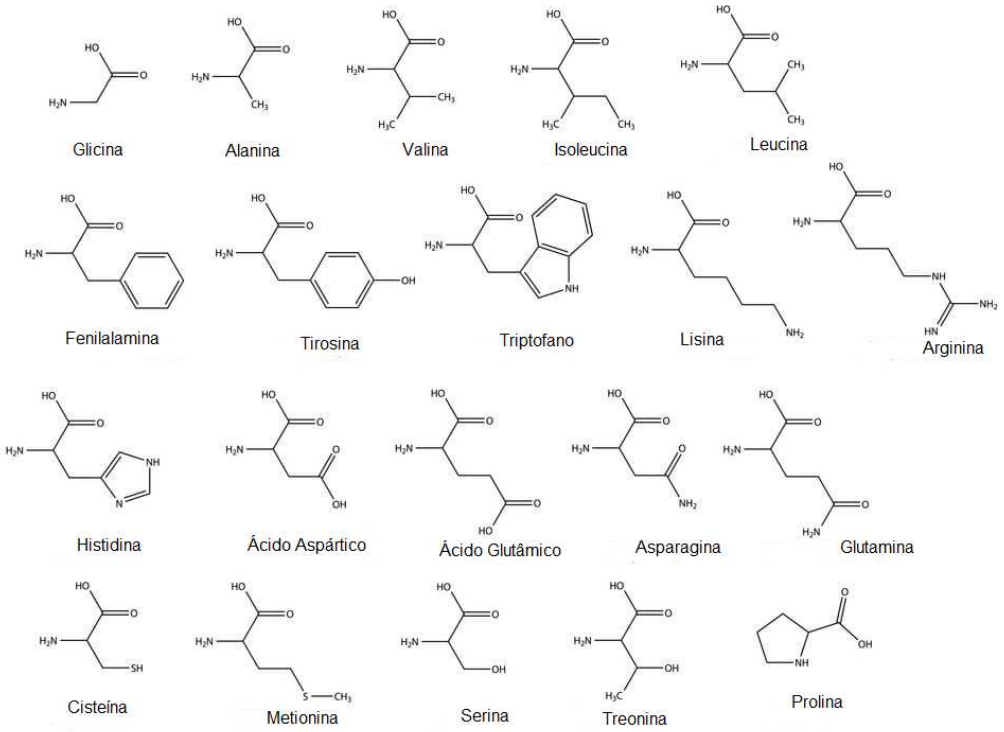

Figura 6. Estrutura química dos aminoácidos protéicos (adaptado de Pure Food Company, 2019).

3.1. Perfil de aminoácidos em cogumelos

Estudos recentes têm investigado o perfil dos aminoácidos presentes em cogumelos. Nestes trabalhos, o teor de aminoácidos foi representado na forma livre (aminoácidos livres) (Mau et al., 1997; Tseng & Mau 1999; Chiang et al., 2006; Tsai et al., 2007; Lee et al., 2009; Kim et al., 2009; ; Beluhan & Ranogajec, 2011; Li et al., 2011; Liu et al., 2012; Kivrak et al., 2014; Li et al., 2014; ; Liu et al., 2014; Ming et al., 2014; Pei et al., 2014; Chen et al., 2015 ; Sun et al., 2017; Dong et al., 2018; Mannimen et al., 2018; Rotola-Pukkila et al., 2019) e total (aminoácidos livres e aminoácidos hidrolisados) (Manzi et al., 1999; Mdachi & Nkunya, 2004; Chirinang & Itarapichet, 2009; Ayaz et al., 2011; Jawoska & Bernas, 2013; Kayode et al., 2015; Fei et al., 2017; Poojary et al., 2017).

Mdachi & Nkunya (2004) identificaram 16 aminoácidos (alanina, ácido aspártico, asparagina, arginina, glutamina, ácido glutâmico, glicina, leucina, lisina, metionina, fenilalanina, serina, treonina, triptofano, tirosina, valine) em cogumelos comestíveis *(Agaricus sp., Boletus pruinatus, Cantharellus cibarius, Lactarius sp., Suillus sp., Pleurotus sajor-caju, Russula hiemisilvae, Inonostus sp., Boletinus cavipes* e *Ganoderma lucidum*) colhidos na região da Tanzânia. Quando comparadas as porcentagens dos aminoácidos essenciais de três espécies de cogumelos (*Lactarius* sp., *Boletinus cavipes* e *Boletus pruinatus*), a leucina foi a mais abundante em todos eles (15,9%, 10,6% e 8,40%, respectivamente). O segundo aminoácido essencial mais abundante foi a valina (11,4%, 7,96% e 6,04%, respectivamente). Treonina foi o terceiro aminoácido essencial mais abundante nestes cogumelos (11,0%, 7,79% e 5,02%, respectivamente). A leucina também foi o aminoácido essencial mais abundante no *Agaricus sp.* (14,2%). *R. hiemisilvae* registrou o menor número e quantidade de aminoácidos essenciais. Das 10 espécies de cogumelos investigadas neste estudo, cinco delas continham de de 2 a 7 dos aminoácidos essenciais em proporções diferentes, ou seja, uma ocorrência de 25% a 88% dos aminoácidos essenciais nos vários cogumelos estudados. Por isso, de acordo com esses autores, os cogumelos colhidos nessa região podem ser uma boa fonte de aminoácidos na alimentação.

Kayode et al. (2015) analisaram aminoácidos em *Pleurotus sajor-caju* (cogumelo ostra) adquirido na Nigéria. Esses autores encontraram os nove aminoácidos essenciais nesses cogumelos e, de acordo com eles, grande parte do

teor dos aminoácidos essenciais encontrados superaram a necessidade diária recomendada para um adulto.

Na Europa e na Ásia concentram-se o maior número de estudos que investigaram a composição de aminoácidos em cogumelos. Beluham & Ranogajec (2011) estudaram a composição de aminoácidos nas 10 espécies mais consumidas pelos croatas (*A. campestris, B. edulis, Calocybe gambosa, Cantharellus cibarius, C. cornucopioides, E. clypeatum, F. velutipes, Macroleptiota procera, M. elata* e *P. ostreatus*). De acordo com esses autores, os cogumelos estudados foram boas fontes de aminoácidos essenciais como leucina, valina, treonina, lisina, metionina e triptofano. Aminoácidos não essenciais como alanina, arginina, glicina, serina e os ácidos glutâmico e aspártico também foram encontrados em grandes quantidades. Os altos teores de ácidos glutâmico e aspárticos estão relacionados ao gosto umami dos cogumelos estudados por esses autores.

Mannimen et al. (2018) estudaram a composição de aminoácidos livres em quatro espécies de cogumelos da *Finnish Forest* na Finlândia (*C. cibarius, C. tubaeformis, B. edulis e L. camphoratus*). Assim como no estudo realizado na Croácia, foi quantificada elevada concentração de aminoácidos relacionados ao gosto umami.

Na Turquia, Kivrak et al. (2014) investigaram os teores de aminoácidos em *Calvatia gigantea,* e foi constatado que, além do uso medicinal, esses cogumelos poderiam ser utilizados na alimentação como fonte de aminoácidos. Também na Turquia, Ayaz et al. (2011) analisaram o teor total de aminoácidos em onze espécies de cogumelos (*L. laccata, L. giganteus, R. rósea, C. cibarius, T. saponaceum, A. arvensis, B. edulis C. rugosa H. repandum C. tubaeformis* e *L. nuda*) colhidas na região. De acordo com esses autores, os cogumelos apresentaram potencial fonte de nutrientes críticos na alimentação, em particular, os aminoácidos essenciais.

Chirinang & Intarapichet (2009) estudaram a composição de aminoácidos em *Pleurotus ostreatus* e *Pleurotus sajor-caju* cultivada na Tailândia. O perfil de aminoácidos foi similar nestas espécies. De acordo com esses autores, as diferenças entre o teor de aminoácidos encontrados na mesma espécie de cogumelos podem ser consequência na variação genética e processo de cultivo aplicados na prática comercial.

Kim et al. (2009) analisaram o teor de aminoácidos em cogumelos comestíveis (*Pleurotus ostreatus, Agaricus bisporus, Flammulina velutipes, Pleurotus eryngii* e *Lentinus edodes*) comercializados na Coreia. De acordo com esses autores, a espécie

A. bisporus apresentou elevado teor de aminoácidos livres e potencial como valor alimentício.

Sun et al. (2017) estudaram os teores de aminoácidos livres em 13 espécies de cogumelos colhidos na província de Yunnan, China (*Boletus bicolor, Boletus speciosus, Boletus sinicus, Boletus craspedius, Boletus griseus, Boletus ornatipes, Xerocomus, Suillus placidus, Boletinus pinetorus, Tricholoma terreum, Tricholomopsis lividipileata, Termitomyces microcarpus* e *Amanita hemilapha*). De acordo com esses autores, os cogumelos apresentaram teores moderados de aminoacidos essenciais, e elevado teor de aminoácidos hidrofóbicos (alanina, prolina, cisteína, valina, metionina, fenilalanina, isoleucina e leucina), os quais podem ser importantes no gosto típico dos cogumelos e apresentam potencial atividade antioxidante.

Outros aminoácidos como ornitina, GABA, citrulina, sarcosina, fosfoserina, taurina, cistationina, etanolamina, hidroxilisina, anserina, metil-histidina, carnosina e hidroxiprolina também foram encontrados em cogumelos (Manzi et al., 1999; Tsai et al., 2007; Kim et al., 2009; Li et al., 2011; Liu et al., 2012; Kivrak et al., 2014; Ming et al., 2014; Poojary et al., 2017).

3.2. Aminoácidos e sua relação com o sabor dos alimentos

A análise de aminoácidos livres em cogumelos como componentes não voláteis para o sabor foi realizada inicialmente por Mau et al. (2001) e Yang et al. (2001). Esses autores dividiram os aminoácidos livres em cogumelos em quatro grupos com base nas características do sabor. O primeiro são os aminoácidos relativos ao gosto umami, similar ao glutamato monossódico, sendo eles, os ácidos glutâmico e aspártico. O segundo grupo apresentou aminoácidos de gosto doce, incluindo alanina, glicina, prolina e treonina. A terceira classe, pertencente aos aminoácidos amargos, inclui a arginina, histidina, isoleucina, metionina, fenilalanina e valina. Lisina e tirosina estão incluidos na quarta classe que é a que não apresenta gosto. Portanto, os aminoácidos umami e doces são os responsáveis pelo gosto agradável dos cogumelos.

Yang et al. (2001) avaliaram o perfil e teor de aminoacidos livres em cinco cogumelos em Taiwan (*F. velutipes*, *L. edodes*, *P. cystidiosus* e *P. ostreatus*). De acordo com esses autores, os cogumelos da espécie *F. velutipes* apresentaram as maiores quantidades de aminoácidos livres totais, enquanto *P. ostreatus*

apresentaram as menores quantidades. A alanina, ácido glutâmico, metionina e treonina foram os quatro aminoacidos livres encontrados em maiores quantidades.

Mau et al. (2001) avaliaram a composição de aminoácidos em seis espécies de cogumelos também comercializados em Taiwan (*Dictyophora indusiata*, *Grifola frondosa*, *Hericium erinaceus* e *Tricholola giganteum*). As quatro espécies de cogumelos apresentaram diferentes perfis e teores de aminoácidos livres. Isoleucina e lisina foram maiores na espécie *Dictyophora indusiata*, alanina e treonina na espécie *Grifola frondosa*, leucina e alanina na espécie *Hericium erinaceus*, e treonina e metionina na espécie *Tricholola giganteum*.

Li et al. (2014) analisaram a composição de 18 aminoácidos no intuito de caracterizar os compostos responsáveis pelo gosto em 5 espécies de cogumelos comercializados na forma desidratada na China (*Agrocybe cylindracea, Pleurotus cystidiosus, Agaricus blazei, Pleurotus eryngii* e *Coprinus comatus*). De acordo com esses autores, *A. blazei, C. comatus* e *A. cylindracea* possuem, relativamente, forte gosto umami (teor dos ácidos glutâmico e aspártico elevados), indicando que esses três cogumelos representam alimentos com gosto intensificado, o que valoriza a importância como possível matéria-prima para o desenvolvimento de flavorizantes para alimentos ou na formulação de alimentos nutracêuticos e funcionais com um gosto umami.

3.3. Fatores que influenciam no perfil de aminoácidos em cogumelos

A influência de variáveis como maturação, diferentes cepas, micélio e corpo de frutificação, diferentes partes do corpo de frutificação foi avaliada por diversos autores. Mau et al. (1997), Tsai et al. (2007) e Ming et al. (2014) estudaram o efeito da maturação dos corpos de frutificação sobre o teor de aminoácidos livres. De acordo com Mau et al. (1997), cogumelos *Volvariela volvacea* mais amadurecidos possuem mais compostos relativos ao gosto do que os no formato ovo e de sino por possuírem maiores teores de compostos do gosto, incluindo os aminoácidos umami. No entanto, os cogumelos *Volvariela volvacea* colhidos nos estágios estipe alongado e chapéu aberto apresentaram taxa mais rápida de senescência e deterioração e, portanto, menor prazo de validade em comparação com os cogumelos em formato de sino e de ovo. No estudo de Tsai et al. (2007), os teores de amino ácidos totais livres em *A. bisporus* diminuíram significativamente com o tempo de maturação. Ming et al. (2014)

estudaram o efeito de três estágios de maturação do cogumelo *Russula grinoula*. Foi possível observar uma diminuição de aminoácidos no terceiro estágio de maturação do cogumelo, com consequente redução no gosto umami que é tanto desejado em cogumelos.

Lee et al. (2009) avaliaram a composição de aminoácidos livres no micélio e no corpo de frutificação de duas cepas de *Hypsizigus marmoreus*. De acordo com esses autores, os corpos de frutificação possuíram compostos de gosto umami intenso principalmente devido aos elevados teores de ácidos glutâmico e aspárticos encontrados, sendo mais elevado que no micélio. Apesar do micélio apresentar teor de aminoácido menor do que o corpo de frutificação, o crescimento do micélio é muito mais rápido, sendo uma alternativa para alimentação. Resultados diferentes foram encontrados por Liu et al. (2012) ao analisarem os teores de aminoácidos em trufas e no micélio de *Tuber sinense, Tuber aestivum, Tuber indicum, Tuber himalayense* e *Tuber borchii var. sphaerospermum*. Nesse estudo, o teor de aminoácidos totais no micélio foi maior que nas trufas, sendo os aminoácidos com gosto umami presentes em maior teor no micélio do que nas trufas. Assim, pelo alto custo e dificuldade de produção de trufas, o micélio é visto como alternativa para alimentação.

Chen et al. (2015) analisaram a composição de aminoácidos no cogumelo *L. edodes* no intuito de avaliar a influência de diferentes partes do cogumelo e maturação na composição de compostos relativos ao gosto. Os resultados mostraram que os teores de treonina (doce) foram os mais elevados entre todos os aminoácidos, com valores mais elevados nas amostras do chapéu que no estipe. O ácido glutâmico (umami) foi o segundo aminoácido livre em maior quantidade, sendo encontrado em teores mais altos nos chapéus do que no estipe, atingindo o valor máximo no último estágio de maturação.

Manzi et al. (1999) estudaram diferentes cepas de *Pleurotus ostreatus* e, de acordo com esses autores, o substrato influencia a composição química e, consequentemente, o valor nutricional dos cogumelos. Dong et al. (2018) estudaram a composição de aminoácidos em 10 lotes diferentes de cogumelos *Lentinula edodes* adquiridos comercialmente em diferentes regiões da China. De acordo com esses autores, a diferença na composição dos aminoácidos pode ser justificada pelas direntes condições climáticas de cada região em que esses cogumelos foram produzidos.

3.4. Influência do processamento e do armazenamento no perfil e teor de aminoácidos em cogumelos

Diferentes condições de estocagem, processamento e técnicas de preservação sobre o perfil e teores de aminoácidos em cogumelos também foram avaliadas. Quando avaliado o efeito do armazenamento, Tseng & Mau (1999) observaram um aumento nos teores de aminoácidos livres após a colheita com o decorrer do armazenamento por 12 dias a 12 ˚C. De acordo com esses autores, cogumelos apresentam taxa de respiração mais elevada que frutas e hortaliças, por serem estruturas reprodutivas. Durante o armazenamento pós-colheita a 12 °C, os açúcares são consumidos pelo processo respiratório para produção de energia e para o desenvolimento pós-colheita, os aminoácidos livres são liberados pela ação das proteases endógenas e os 5'-nucleotideos são liberados pela ação das enzimas que degradam os ácidos nucleicos.

Chiang et al. (2006) analisaram a composição de aminoácidos em três espécies de cogumelos enlatados (*Agaricus bisporus, Volvariella volvacea e Flammulina velutipes*) adquiridos comercialmente em Taiwan. Os principais aminoácidos encontrados no *A. bisporus* foram lisina, ácido glutâmico e alanina, no *V. volvacea* foram lisina, alanina e no *F. velutipes* foram arginina, tirosina, fenilalanina e ácido glutâmico. Grandes perdas de aminoácidos umami e 5’-nucleotídeos nos cogumelos enlatados ocorreram durante o processamento, especialmente no estágio de branqueamento. Portanto, no que diz respeito ao conteúdo de componentes de gosto, os cogumelos enlatados apresentam gosto menos atraente e não comparáveis aos cogumelos frescos cozidos.

Rotola-Pukkila et al. (2019) além de estudarem o perfil e o teor em diferentes espécies, estudaram também o efeito do cozimento nessas espécies. Nesse estudo foi possível observar que a soma dos teores liberados no caldo de cozimento e no cogumelo cozido é superior ao determinado no fresco.

Liu et al. (2014) investigaram o efeito de técnicas de preservação (congelamento, enlatamento e salga) em *Agaricus bisporus*. Os cogumelos congelados apresentaram maior teor de aminoácidos livres que os enlatados e os submetidos à salga. De acordo com os autores, a redução de aminoácidos livres pode ser resultado do escurecimento não enzimático (reação de Maillard) em decorrência do tratamento térmico, a qual envolve a reação entre grupos amino dos aminoácidos

e os grupos carboxila dos açúcares. Li et al. (2011) avaliaram o efeito de diferentes processamentos nos teores de aminoácidos em sopas de cogumelo *A. bisporus*: autoclave, fervura e micro-ondas. O tratamento por micro-ondas foi o que apresentou a maior capacidade de preservar os aminoácidos nas sopas de cogumelos, reforçando a influência da reação de Maillard na perda de aminoácidos.

De acordo com Jawoska & Bernas (2013), o processo de congelamento seguido de armazenamento congelado diminuiu os teores de aminoácidos livres nos cogumelos congelados quando comparado aos cogumelos frescos.

Pei et al. (2014) avaliaram o efeito dos métodos não convencionais de processamento (liofilização e liofilização por micro-ondas) na desidratação de *Agaricus bisporus*, não observando diferenças significativas. Fei et al. (2017) avaliaram o efeito da desidratação osmótica convencional e por ultrassom nos teores de aminoácidos em cogumelos *A. bisporus* e observaram que por ultrassom foi mais eficiente que da forma convencional, retendo mais os aminoácidos. A influência da desidratação por pulso nos teores de aminoácidos livres em cogumelos das espécies *Cantharellus cibarius* e *Armillariella melleathe* foi avaliada por Shcheglova & Vereshchagin (2015). Foi observado que a desidratação por pulso a vácuo promoveu o acúmulo de aminoácidos livres nesses cogumelos, aumentado o valor nutricional desse alimento.

Com relação ao valor nutricional, a análise de aminoácidos livres subestima o valor nutricional dos cogumelos por não considerarem a presença dos aminoácidos que estão ligados, como os peptídeos e as proteínas. Da mesma forma, a hidrólise total pode superestimar o valor nutricional, uma vez que pode não corresponder ao teor de aminoácidos liberados durante a digestão em organismo vivo, muito menos a quantidade que é absorvida. Contudo, pelos teores de aminoácidos livres e hidrolisados, é possível ter ideia sobre o potencial nutritivo dos cogumelos.

Apesar de boa parte dos trabalhos citados identificarem a presença de aminoácidos essenciais nos cogumelos, o elevado teor de umidade e a pequena quantidade de proteínas usual em cogumelos, comprometem a classificação dos cogumelos (Kalač, 2013) como fonte de proteínas e aminoácidos essenciais (Manzi et al., 1999; Mdashi & Nkunya, 2004; Kayode et al., 2015). Contudo, métodos de preservação como a desidratação podem prover a quantidade de aminoácidos essenciais necessários para alimentação (FAO, 2007), encontrados comumente em cogumelos desidratados (Li et al., 2011; Li et al., 2014; Dong et al., 2018).

3.5. Métodos de determinação de aminoácidos em cogumelos

São diversas as técnicas disponíveis para a análise de aminoácidos em cogumelos, dentre elas a cromatografia líquida, a cromatografia gasosa e o uso de analisadores de aminoácidos (Tabelas 4 e 5).

O preparo de amostra para análise de aminoácidos difere com relação ao objetivo em cada trabalho. Na grande maioria dos trabalhos visando a determinação da composição de aminoácidos livres em cogumelos, foi utilizada a extração ácida por HCl na concentração de 0,1 mol/L (Mau et al., 1997; Tseng & Mau, 1999; Chiang et al., 2006; Tsai et al., 2007; Lee et al. 2009, Liu et al., 2014; Li et al., 2014).

A extração por ultrassom utilizando HCl 0,1 mol/L foi aplicada por Chen et al. (2015), usando banho ultrassom por 30 min a 40 °C. Sun et al. (2017) utilizaram também a técnica de extração por ultrassom, mas usaram metanol 80% seguido do banho ultrassom. Chen et al. (2015) e Li et al. (2011) utilizaram solução de ácido tricloroacético 10% como extrator. Manninen et al. (2018) e Rotola-Pukkila et al. (2019) utilizaram apenas a água como solvente extrator em temperatura de ebulição em banho de ultrassom para extração de aminoácidos.

Pei et al. (2014) utilizaram a técnica de separação em fase sólida (SPE) para separação dos aminoácidos, com água como solvente extrator a 90 °C.

Na extração de aminoácidos livres de cogumelos para posterior análise por cromatografia líquida de alta eficiência com detecção por espectrometria de massas (HPLC–MS) (Kivrak et al., 2014; Ming et al., 2014; Dong et al., 2018), o preparo de amostra tem sido mais elaborado. Ming et al. (2014) extraíram as amostras de cogumelos com metanol:água (1:1) e adicionaram ácido sulfossalicílico até 10%. A amostra foi misturada e centrifugada. O sobrenadante foi misturado com padrão interno (norvalina). As amostras foram novamente agitadas e centrifugadas e o sobrenadante diluído dessa nova separação foi misturado com 5 µL de reagente iTRAQ® para derivatização. A amostra foi incubada em temperatura ambiente por 30 min, evaporada e reconstituídas para análise.

Dong et al. (2018) utilizaram amostras de cogumelos secos. A amostra foi suspensa em água deionizada e agitada por 1 min a 80 °C. Após centrifugação, o sobrenadante foi diluído para uma concentração apropriada e o padrão interno foi adicionado. A desproteinização foi realizada pela adição de metanol gelado na proporção de 1:8 (v/v) o extrato foi incubado a 4˚C durante 10 min. Depois de

centrifugado, o sobrenadante foi recolhido e evaporado. O resíduo foi redissolvido com ácido fórmico em água 0,1% (v/v) e centrifugado antes da injeção.

Dentre os métodos disponíveis na literatura que fizeram a análise de aminoácidos livres por cromatografia liquida acoplada a espectrometria de massas, o preparo de amostra mais simples foi apresentado por Kivrak et al. (2014), em que a amostra foi pesada e extraída com de ácido fórmico a 0,1% (v/v) em solução água: metanol (80:20) (v/v). A mistura foi agitada, centrifugada e o sobrenadante foi injetado.

Os trabalhos que propuseram a análise de aminoácidos totais (livres e ligados) tiveram que adotar a hidrolise proteica. O ácido clorídrico, assim como nos métodos de extração de aminoácidos livres, também foi utilizado, contudo na concentração de 6 mol/L, a 110 ˚C durante 24 horas sob fluxo gás nitrogênio (Chirinang & Intarapichet, 2009; Ayaz et al., 2011; Jawoska & Bernas, 2013. Fei et al. (2017) também empregaram essa técnica pela hidrólise ácida a 110 °C durante 24 horas, contudo o extrato foi concentrado por auxilio de evaporador rotatório. Alguns cuidados adicionais foram adotados no processo de hidrólise: Jawoska & Bernas (2013) adicionaram fenol para proteção da tirosina e Chirinang & Intarapichet (2009) fizeram hidrólise alcalina com NaOH para análise.

Poojary et al. (2017) compararam a extração convencional de aminoácido em amostras de cogumelos (HCl 0,1 mol/L, por 45 min) com os métodos de extração enzimáticos. Observou-se que a aplicação de enzimas para extração de aminoácidos em cogumelos (Shitake, Ostra, Champignon, Champignon escuro e Portobello) aumentou a capacidade de extração de aminoácidos de sabor umami e forneceu maior rendimento.

Tabela 4. Métodos de preparo da amostra para determinação de aminoácidos em cogumelos.

Nº aminoácidos	Preparo da amostra	Referência
13	Pó liofilizado (0,5 g) foi agitado com 50 ml de HCl 0,1 mol/L durante 45 min.	Mau et al., 1997
20	Não informado.	Manzi et al., 1999
18	Pó liofilizado (0,5 g) foi agitado com 50 ml de HCl 0,1 mol/L durante 45 min.	Tseng & Mau 1999
22	O cogumelo seco foi embebido em água destilada por 24 horas e o extrato resultante foi filtrado.	Mdachi & Nkunya, 2004
18	Pó liofilizado (0,5 g) foi agitado com 50 ml de HCl 0,1 mol/L durante 45 min.	Chiang et al., 2006
17	Pó liofilizado (0,5 g) foi agitado com 50 ml de HCl 0,1 mol/L durante 45 min.	Tsai et al., 2007
18	Cogumelo seco foi hidrolisado sob nitrogênio gasoso com HCl 6 mol/L em autoclave a 110 ˚C durante 24 horas. Em seguido, neutralizada a pH 7,00 com NaOH 4 M. A hidrólise alcalina foi feita para análise de triptofano.	Chirinang & Intarapichet, 2009
17	Pó liofilizado (0,5 g) foi agitado com 50 ml de HCl 0,1 mol/L durante 45 min, em seguida foi filtrado e misturado com ortoftaladeído.	Lee et al., 2009
18	Cogumelo em pó foi hidrolisado em HCl 6 mol/L contendo fenol a 0,1% (p/v) a 110 ˚C por 24 horas sob vácuo.	Ayaz et al., 2011
17	Não informado.	Beluhan & Ranogajec, 2011
20	Sopas de cogumelos liofilizados foram extraídas com ácido tricloroacético a 10% (p/v) durante 2 horas em temperatura ambiente e a mistura e centrifugada a durante 10 min. Este filtrado foi misturado com o reagente ortoftalaldeído.	Li et al., 2011
20	Pó liofilizado (0,5 g) foi agitado com 50 ml de HCl 0,1 mol/L durante 45 min.	Liu et al., 2012
18	Amostras em pó foram realizadas em HCl 6 mol/L contendo 0,5% de fenol (para proteção contra tirosina) a 110 ˚C por 24 horas sob atmosfera de argônio.	Jawoska & Bernas, 2013
21	Amostra foi extraída em ácido fórmico a 0,1% (v/v) e solução água: metanol (80:20) (v/v). A mistura foi agitada com e depois imediatamente centrifugada.	Kivrak et al., 2014
18	Amostra liofilizada foi extraída com 0,1 HCL por 45 min e filtrada. O filtrado foi misturado com ácido 5-sulfosalicílico 8% e centrifugado.	Li et al., 2014
21	O pó de cogumelo liofilizado (500 mg) foi diluído com 50 ml de HCl 0,1 mol/L. A amostra foi mantida à temperatura ambiente durante 45 min e depois centrifugada a 10 000 g durante 15 min.	Liu et al., 2014
42	A amostra de cogumelo foi extraída com metanol / água. Foi adicionado ácido sulfosalicílico a 10%. A amostra foi misturada e centrifugada. O sobrenadante foi misturado com tampão borato (contendo padrão interno norvalina). O sobrenadante foi misturado e centrifugadas. E o sobrenadante foi diluído, misturado com reagente iTRAQ® para marcação e incubados à temperatura ambiente por 30 min. Em seguida, hidroxilamina a 1,2% foi adicionada. As amostras foram evaporadas durante a noite e foram reconstituídas com 3 de mistura padrão rotulada com reagente iTRAQ®.	Ming et al., 2014
14	Amostras secas e moídas foram extraídas com de água destilada a 90 ˚C por 20 min. O extrato foi centrifugado e o sobrenadante foi filtrado e submetidos a extração em fase sólida, filtrado em cartucho de SPE-PAK-C18. Os compostos retidos foram eluídos e o eluato resultante foi recolhido, evaporado, redissolvido em água destilada, filtrado e misturado com o reagente ortoftalaldeído	Pei et al., 2014
21	Amostras seca foi extraída em HCl 0,1 mol/L e coloca em banho de ultrassom a 40 ˚C. A amostra foi centrifugada com ácido sulfossalicílico a 40 ˚C. O sobrenadante foi neutralizado a pH = 2.0 com NaOH:HCl.	Chen et al., 2015
18	Cogumelo seco foi extraído por Soxhlet com éter de petróleo. O extrato foi hidrolisado três vezes, o teor de aminoácido foi extraído com diclorometano, concentrado e derivatizado para volatilidade.	Kayode et al., 2015
17	Não informado.	Liu et al., 2016

Tabela 4. Métodos de preparo da amostra para determinação de aminoácidos em cogumelos (continuação).

Nº aminoácidos	Preparo da amostra	Referência
18	A amostras secas foi hidrolisada com HCl 6 mol/L, a 110 ˚C durante 24 h. As amostras foram concentradas usando um evaporador rotativo e o resíduo foi dissolvido em HCL 0,02 mol/L.	Fei et al., 2017
20	Cogumelo liofilizado foi extraído por etanol a 75% (v/v) a 80 ˚C por 15 min. Em seguida foi centrifugado. Os sobrenadantes foram concentrados a 55 ˚C sob pressão reduzida e liofilizados sucessivamente. Os resíduos sólidos foram dissolvidos em solução de ácido tricloroacético a 3% com ultrassom. As soluções foram centrifugadas e os sobrenadantes foram filtrados.	Sun et al., 2017
19	Amostras de cogumelos secos foram extraídas em 50 mL de HCl 0,1 mol/L durante 45 min. Extração por enzimas – Foi utilizado a beta-glucanase, *Flavourzyme* ou mistura 1:1 v/v de beta-glucanase e *Flavourzyme*). A solução com enzima foi pré-aquecida. A amostra de cogumelo foi então adicionada no frasco e agitada durante a duração requerida. No final do processo de extração, a agitação foi interrompida e o extrato foi filtrado através de um papel de filtro sob vácuo. Preparação da amostra – Foi adicionado padrão interno (norvalina). A solução foi aspirada suavemente através de uma ponta sorvente de permuta catiônica (extração em fase sólida). As partículas de sorvente foram então lavadas com propanol a 33%. Os aminoácidos foram então eluídos em uma mistura de eluição consistindo em NaOH a 0,33 mol/L, propanol a 80% e 3-picolina a 20%. Os aminoácidos foram então derivatizados utilizando como agente derivatizante composto por mistura 2: 6: 2 v/v/v de cloroformato de propila/ clorofórmio / iso-octano. A mistura resultante foi em seguida submetida a agitação durante 1 e foram adicionados uma mistura 90:10 v/v de isso-octano / clorofórmio. A solução foi agitada e foram adicionados HCl 1 mol/L. A solução foi agitada o que permitiu separar em duas fases. A camada superior contendo aminoácidos livres derivatizados foi transferida para uma inserção de GC para análise.	Poojary et al., 2017
20	Os cogumelos secos foram moídos em pó fino. A amostra foi suspensa em água deionizada e agitados a 80 ° C. Em seguida centrifugada e o sobrenadante foi diluído e o padrão interno foi adicionado. A desproteinização foi realizada pela adição de metanol gelado 1:8 (v/v). O extrato centrifugado e o sobrenadante foi recolhido e evaporado. O condensado foi redissolvido em de ácido fórmico a 0,1%, centrifugado e o sobrenadante foi injetado.	Dong et al., 2018
26	Cogumelo liofilizado foi extraído em água fervente (100 ˚C) e mantidas em banho de ultrassom a 23 ˚C. As amostras foram centrifugadas e o sobrenadante foi coletado.	Manninen et al., 2018
26	Cogumelo liofilizado foi extraído em água a 100 ˚C e banho ultrassônico. Em seguida foi centrifugação e o sobrenadante injetado.	Rotola-Pukkila et al., 2019

Tabela 5. Métodos de detecção e quantificação de aminoácidos em cogumelos.

Nº aminoácidos	Sistema/Fase estacionária	Derivatização/ Detecção	Tempo de corrida	Referências
13	HPLC Hitachi F-1050 Coluna C18 Prodigy 5 ODS-2 (4,6 x 250 mm, 5 µm, Phenomenex)	Pré-coluna com ortoftaladeído Detecção por fluorescência (excitação 340 nm, emissão 450 nm)	43 min	Mau et al., 1997
20	Analisador de aminoácidos Beckman 120C com coluna de troca iônica (32 x 0,9 cm) HPLC Waters model 501 com coluna C18 (250 mm x 5 µm, Supelco) Espectrofluorímetro modelo LS 40 usado para análise de triptofano	Derivatização pós-column com niridrina Detecção na região visível	Não informado	Manzi et al., 1999
18	HPLC Hitachi F-1050 Coluna C18 Prodigy 5 ODS-2 (4,6 x 250 mm, 5 µm, Phenomenex)	Derivatização pré-coluna com ortoftaladeído Detecção por fluorescência (excitação 340 nm, emissão 450 nm)	43 min	Tseng & Mau, 1999
22	HPLC modelo SHIMADZU LC-10AT Coluna SRT ODSM (4,6 x 150 mm)	Derivatização pré-coluna com ortoftaladeído Detecção por fluorescência (excitação 340 nm, emissão 450 nm)	Não informado	Mdachi & Nkunya, 2004
18	HPLC Coluna C18 LiChrospher 100 RP-18 (4,6 x 250 mm, 5 µm, Merck)	Derivatização pré-coluna com ortoftaladeído Detecção por fluorescência (excitação 340 nm, emissão 450 nm)	43 min	Chiang et al., 2006
17	HPLC Coluna C18 Synergi 4µ Fusion-RP 80 (4,6 x 250 mm, 4 µm, Phenomenex)	Derivatização pré-coluna com ortoftaladeído Detecção por fluorescência (excitação 340 nm, emissão 450 nm)	43 min	Tsai et al., 2007
18	HPLC Agilent modelo 1100 Coluna Zorbax Eclipse XDB-C18 (4,6 x 150 mm, 5 µm)	Derivatização com ortoftaldeído e ácido 3-mercaptopropionico para aminoácidos primários e cloroformiato de 9-fluorenilmetila para aminoácidos secundários Detecção por fluorescência	Não informado	Chirinang & Intarapichet, 2009
17	HPLC Hitachi modelo L-7485 Coluna C18 LiChrospher 100 RP-18 column (4,6 x 250 mm, 5 µm, Merck)	Derivatização pré-coluna com ortoftaladeído Detecção por fluorescência (excitação 340 nm, emissão 450 nm)	43 min	Lee et al., 2009
18	Analisador de aminoácidos modelo Hitachi 8800	Derivatização pós-coluna com niridrina Detecção na região visível	Não informado	Ayaz et al., 2011
17	HPLC Coluna Zorbax Eclipse-AAA (3,0 x 150 mm, 3,5 µm)	Derivatização pré-coluna com ortoftaldeído e ácido 3-mercaptopropionico Detecção por fluorescência (excitação 340 nm, emissão 450 nm) Detecção por deteccão de arranjo de diodos a 338 nm para aminoácidos primários e a 262 nm para aminoácidos secundários	26 min	Beluhan & Ranogajec, 2011

Tabela 5. Métodos de detecção e quantificação de aminoácidos em cogumelos (continuação).

Nº aminoácidos	Sistema/Fase estacionária	Derivatização/ Detecção	Tempo de corrida	Referências
20	HPLC modelo Agilent 1100 Coluna C18 Diamonsil (4,6 x 250 mm)	Pré-coluna com ortoftalaldeído Detecção não informada	Não informado	Li et al., 2011
20	Analisador de aminoácidos modelo Hitachi 835-50	Derivatização pós-coluna com niridrina Detecção na região visível	Não informado	Liu et al., 2012
18	Analisador de aminácidos	Derivatização pós-coluna com niridrina Detecção na região visível	Não informado	Jawoska & Bernas, 2013
21	UPLC–MS/MS modelo Waters Xevo TQ-S triplo quadrupolo equipado com ionização por eletro spray (ESI) Coluna C18 Waters Acquity UPLC BEH (2,1 x 100 mm, 1.7 µm)	Detecção por espectrometria de massas	10 min	Kivrak et al., 2014
18	Cromatografia de troca iônica de alta performance Coluna de troca iônica modelo Pac PA-10 (2,0 x 250 mm). (HPAEC-PAD)	Detecção por pulso amperometrico	Não informado	Li et al., 2014
21	Analisador de aminoácidos modelo L-8800	Derivatização pós-coluna com niridrina Detecção na região visível	Não informado	Liu et al., 2014
42	HPLC Coluna C18 (4,6 x 150 mm, 5 µm)	Detecção por espectrometria de massas (triplo quadrupolo Biosystems/SCIEX 3200QTRAP (USA))	25 min	Ming et al., 2014
14	HPLC modelo Agilent 1200 Coluna C18 Zorbax Eclipse XDB (4,6 x 250 mm, 5 µm, Agilent)	Pré-coluna com ortoftalaldeído Detecção UV	35 min	Pei et al., 2014
21	Analisador de aminoácido modelo L-8800 Hitachi	Derivatização pós-coluna com niridrina Detecção na região visível	Não informado	Chen et al., 2015
18	Cromatógrafo gasoso com detector de massas modelo Agitent 5973	Detecção por espectrometria de massas	Não informado	Kayode et al., 2015
17	HPLC modelo Agilent 1100 Coluna C18 Hypersil ODSC18 (4,0 x 125 mm, Agilent)	Derivatização pré-coluna com ortoftalaldeído Detector UV modelo Waters 2478 a 338 nm, ou 262 nm para prolina.	24 min	Liu et al., 2016
18	Analisador de aminoácidos Hitachi	Derivatização pós-coluna com niridrina Detecção na região visível	Não informado	Fei et al., 2017
19	Cromatógrafo gasoso modelo - Agilent 6890 (Agilent, USA) Coluna ZB–AAA column (0,25 x 100 mm, 0,25 µm film)	Detecção por ionização em chamas (FID)	Não informado	Poojary et al., 2017

Tabela 5. Métodos de detecção e quantificação de aminoácidos em cogumelos (continuação).

Nº aminoácidos	Sistema/Fase estacionária	Derivatização/ Detecção	Tempo de corrida	Referências
20	HPLC Coluna ZORBAX Eclipse-AAA column (3,0 x 150 mm, 3,5 µm, Agilent)	Derivatização pré-coluna com ortoftalaldeído Detecção por fluorimetria a 340 nm excitação, 450 nm emissão e troca de sinal para 266 nm de excitação e 305 nm emissão	26 min	Sun et al., 2017
20	HPLC Coluna C18 AB Sciex AAA (4.6 x 150 mm, 5 µm)	Detecção por espectrometria de massas (AB Sciex, 4000 Qtrap System, America)	18 min	Dong et al., 2018
26	UHPLC Coluna modelo C18 100 (Phenomenex)	Derivatização pré-coluna com ortoftalaldeído e ácido 3-mercaptopropionico Detecção por fluorimetria a 340 nm excitação, 450 nm emissão e troca de sinal para 266 nm de excitação e 305 nm emissão	25 min	Manninen et al., 2018
26	UHPLC Coluna C18 Kinetex (4,6 x 100 mm, 2,6 µm; Phenomenex)	Derivatização pré-coluna com ortoftalaldeído e ácido 3-mercaptopropionico Detecção por fluorimetria a 340 nm excitação, 450 nm emissão e troca de sinal para 266 nm de excitação e 305 nm emissão.	25 min	Rotola-Pukkila et al., 2019

Com relação às técnicas de análise para separação e detecção de aminoácidos em cogumelos, no geral, os trabalhos podem ser separados em métodos que utilizaram da derivatização pós-coluna, pré-coluna, ou detecção por espectrometria de massas. Assim como as aminas bioativas, os aminoácidos são substâncias altamente ionizáveis, dessa forma, apresentam elevada polaridade e, portanto, são separadas com melhor eficiência em colunas de troca iônica, tendo baixa eficiência em uma coluna C18. Equipamentos conhecidos como analisadores de aminoácidos apresentam como princípio de separação a cromatografia de troca iônica, derivatização pós-coluna por niridrina e detecção por espectrometria na região do visível (Friedman, 2004). Essa técnica foi empregada por diversos autores para análise de aminoácidos (Manzi et al., 1999; Ayaz et al., 2011; Li et al., 2011; Liu et al., 2012; Jawoska & Bernas, 2013; Liu et al. 2014; Chen et al., 2015; Fei et al., 2017). Assim como nos analisadores de aminoácidos, Li et al. (2014) utilizaram o princípio de separação de aminoácidos por troca iônica, entretanto a detecção dos aminoácidos foi por pulso amperométrico.

A derivatização pré-coluna possibilita alteração na estrutura química dos aminoácidos de forma a oferecer um grupo hidrofóbico e, consequentemente, desenvolver afinidade do derivatizado com a fase reversa, melhorando assim a separação e o tempo de corrida. Dessa forma diversos autores utilizaram da técnica de cromatografia liquida de alta eficiência em separação por fase reversa para determinação de aminoácidos, utilizando a derivatização pré coluna a partir da reação com ortoftaladeído, em que a detecção pode ser na região do ultravioleta (Pei et al., 2014; Liu et al., 2016) ou por fluorescência (Mau et al., 1997; Mdachi & Nkunya, 2004; Chiang et al., 2006; Tsai et al., 2007; Chirinang & Intarapichet, 2009; Lee et al., 2009; Beluhan & Ranogajec, 2011; Sun et al., 2017; Manninen et al., 2018; Rotola-Pukkila et al., 2019).

Com o avanço da técnica cromatografia líquida acoplada a espectrometria por massas, a separação dos aminoácidos deixa de ser um desafio, sendo possível determinar uma maior variedade de aminoácidos em curto espaço de tempo utilizando o princípio de separação por fase reversa. Ming et al. (2014) registraram a quantificação de 42 aminoácidos em 25 minutos. Dong et al. (2018) quantificaram 21 aminoácidos em 18 min. Kivrak et al. (2014), por utilizar da técnica de separação por ultra eficiência (UHPLC), conseguiram determinaram 21 aminoácidos em cogumelos em apenas 10 minutos. Diferente dos demais trabalhos, Poojary et al. (2007)

utilizaram a técnica de separação de aminoácidos por cromatografia gasosa em coluna capilar, utilizando a detecção por ionização de chama (FID). Essa técnica de separação aumenta o preparo de amostra visando a necessidade de derivar aminoácidos, a fim de aumentar a volatilidade e serem arrastados pela fase móvel.

Os métodos de análise de aminoácidos em cogumelos se basearam, em sua grande maioria, na extração ácida para análise de aminoácidos livres por ácido forte ou fraco diluídos e na extração prolongada com ácido forte concentrado para hidrólise proteica. Os trabalhos que utilizaram a análise por derivatização pós-coluna através dos analisadores de aminoácidos, em que incluem a separação por troca iônica, apresentaram tempo elevado na separação, uma vez que a elevada característica polar dessas substâncias faz com que a corrida seja longa e as trocas iônicas na fase estacionária sejam suficientes para separação das substâncias que posteriormente são derivadas por niridrina. A adoção do ortoftalaldeído para derivatização pré-coluna foi um avanço analítico que permitiu a aplicação de colunas C18 (fase reversa) para separação de aminoácidos. Esse método possibilita a diversificação dos métodos para análise de aminoácidos por variedade de equipamentos como HPLC e UPLC nos quais, além dos aminoácidos, podem ser analisadas diversas classes de analitos ao contrário dos analisadores de aminoácidos que se destinam unicamente a esse fim. Contudo, por se tratar de um método de derivatização pré-coluna, o preparo da amostra torna-se mais elaborado e complicado, aumentando a influência do analista no viés do método quando comparado ao método pós-coluna.

O uso de cromatografia gasosa, apesar de apresentar uma boa resolução na separação por coluna capilar, apresenta preparo de amostra bem dispendioso. Contudo qualifica mais uma técnica adicional para análise de aminoácidos em cogumelos. A análise de aminoácidos por cromatografia líquida acoplada a espectrometria massas, reduz a necessidade de separação pela fase estacionária, todavia, uma vez que os cogumelos são uma matriz complexa, o uso desse método requer um preparo de amostra mais complexo tendo em vista a necessidade de redução de interferentes. Além disso, a aplicação dessa técnica sofisticada apenas para análise de aminoácidos proteicos que estão na concentração de mg/100 g em alimentos, representa um exagero analítico. Contudo, a cromatografia com detecção por espectrometria de massas pode ser fundamental para a determinação de aminoácidos não proteicos que foram encontrados em pequenas quantidades em

alimentos e podem ser um recurso no desenvolvimento de padrões de identidade e qualidade.

4. Perspectivas de estudos

A importância de duas classes de analitos presente nos cogumelos foi destacada. As aminas bioativas e os aminoácidos livres são compostos que ocorrem naturalmente em uma variedade de alimentos e exercem importantes funções no metabolismo humano como blocos de construção de proteínas (Marchini et al., 2016) ou estabilizadores do DNA and RNA (Kalač, 2014). Os cogumelos se classificam pelo elevado teor de espermidina, enquanto apresentam potencial para formação de outras aminas bioativas pela descarboxilação de aminoácidos livres. O efeito do processamento na composição de aminoácidos em cogumelos foi estudado e é possível constatar a influência significativa desses nos teores de aminoácido dos cogumelos. Todavia, são escassos os estudos que analisaram o efeito do processamento nas poliaminas e outras aminas bioativas. Apenas dois estudos na literatura (Yen, 1992; Kalač & Krizek, 1997) investigaram o efeito do processamento sobre os teores de aminas biogênicas (triptamina, feniletilamina, putrescina, cadaverina, histamina e tiramina) em cogumelos. Métodos analíticos que determinam ambos componentes (aminas bioativas e aminoácidos) tornam-se de particular interesse, não apenas para fornecer informação nutricional ou qualidade higiênica, mas também por possibilitar o monitoramento do processo (Fiechter et al., 2013).

O processamento de alimentos e as condições de armazenamento podem causar mudanças em ambos os teores de aminoácidos e aminas biogênicas. Essas alterações podem ser usadas para avaliar os tempos de amadurecimento ou como indicadores para refletir o grau de frescor ou deterioração do produto alimentício (Jia et al., 2011). Além disso, o perfil e o teor de aminoácidos livres influenciam o gosto do alimento. Não somente os aminoácidos, mas tanto a putrescina quanto a cadaverina podem contribuir com o sabor putrefativo aos alimentos (Wang et al., 1975).

Apesar dos diversos estudos que abordaram o valor nutricional dos cogumelos, é fato que a extração para aminoácidos livres e ou hidrólise proteica podem subestimar e superestimar, respectivamente, o valor nutricional dos cogumelos. No primeiro caso, são excluídos os teores de aminoácidos ligados e peptídeos que serão liberados pelas enzimas digestivas no trato gastro intestinal. No segundo caso,

considera-se que toda proteína no cogumelo será hidrolisada. Dessa forma, para conhecer o valor nutricional, é necessário conhecer, além do perfil, os teores de aminoácidos livres e ligados que serão liberados pelas proteínas durante digestão no trato gastrointestinal.

A investigação da bioacessibilidade é uma ferramenta que deve ser aplicada quando se quer saber a porção das substâncias ingeridas na dieta que são liberadas no trato intestinal após a digestão. Por se tratar de um alimento com elevado teor de espermidina e de outras aminas, o conhecimento da bioacessibilidade de aminas ao longo do trato gastrointestinal e sua liberação da matriz de cogumelo, podem ajudar a esclarecer o impacto dos cogumelos provenientes da dieta na saúde humana.

A bioacessibilidade é definida como a quantidade de um nutriente ingerido que está disponível para absorção no intestino após a digestão e pode ser determinada por métodos *in vivo* e *in vitro*. Os métodos *in vivo* provêm dados diretos e envolvem animais e humanos, o que representa alto custo e impedimentos éticos (Guerra et al., 2012, Minekus et al., 2014). Dessa forma, a abordagem *in vitro* torna-se preferencial por seu baixo custo e ausência de constrangimentos éticos.

Junto com a bioacessibilidade, o efeito do processamento de alimentos deve ser investigado, uma vez que pode ter muitos efeitos positivos ou negativos na bioacessibilidade de compostos bioativos durante a passagem pelo trato gastrointestinal. O impacto do processamento na bioacessibilidade dos compostos bioativos tem sido indicado não apenas como uma simples relação causa-efeito. Apesar do aumento da palatabilidade e da segurança alimentar através do processamento, compostos nativos e nutrientes, que são essenciais para a dieta, muitas vezes podem ser perdidos no processo, diminuindo assim sua biodisponibilidade (Ariza et al., 2018; Cilla et al., 2018; Mercadante & Mariutti, 2018).

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CAPÍTULO II: Optimization and validation of UHPLC method for multianalysis of free amino acids and bioactive amines in mushrooms

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Abstract

The simultaneous determination of 17 free amino acids and 10 biologically active amines in *Agaricus bisporus* mushroom was investigated for the first time. The extraction procedure was optimized; a Plackett-Burman design was used to screen the main factors that could affect recovery and a Central Composite Rotational Design evaluated the interactions between variables. The extracted analytes were derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (Accq) prior to separation and quantification by a RP-UHPLC method and UV detection. The method was validated according to the European Community guidelines [1] (EC, 2002). Optimal conditions for the extraction of the amines, amino acids and ammonium ion in mushrooms were set (2 successive extractions, 2 min shaking at 200 rpm, 4 min centrifugation at 7,000 *g* and 1.6% TCA). The chromatographic conditions were optimized to provide the shortest possible run of all analytes with appropriate resolution. The run had a total time of 15 min and all analytes eluted within 12 min. LOD and LOQ varied from 0.04 to 0.58 mg/100 g and 0.14 to 1.92 mg/100 g, respectively. The mean recoveries for most analytes were between 80% and 110%. The repeatability for most analytes had a CV below 10% and reproducibility below 15%. Only spermidine was detected at mean levels of 7.7 and 5.6 mg/100 g in fresh and canned mushrooms, respectively. Concerning the 17 amino acids analyzed, only methionine and cystine were not detected in the mushrooms. Total mean levels of amino acids were 460.3 and 144.3 mg/100 g in fresh and canned mushroom, respectively. Alanine and glutamic acid were the predominant amino acids (~20%), followed by proline (~14%) of total levels.

Keywords: Polyamines; 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate; Plackett–Burman; *Agaricus bisporus.*

1. Introduction

Nitrogenous compounds, among them amino acids and biologically active amines are naturally occurring compounds that are inherent to a variety of food, including mushrooms. Amino acids are subunits of proteins, making a total of 20 different substances, classified according to the human nutritional need as essential, non-essential and conditionally essential. Essential amino acids are indispensable for vital functions since they are involved in the biosynthesis of enzymes, hormones and structural proteins. The absence or inadequate ingestion of essential amino acids in the diet can cause a negative nitrogen balance, which can lead to weight loss, impaired growth, and other clinical symptoms [2]. Amino acids are also relevant contributors to food taste [3, 4, 5]. For example, aspartic and glutamic acids are considered umami, monosodium glutamate-like (MSG-like) or palatable taste amino acids. Some amino acids impart a sweet taste, e.g. alanine, glycine, proline, serine and threonine; whereas others are considered bitter amino acids, among them arginine, histidine, isoleucine, leucine, methionine, phenylalanine, and valine. Lysine and tyrosine are tasteless amino acids.

In addition, amino acids are the precursors of bioactive amines, which can be formed by decarboxylation of amino acids, induced by heat or by microbial enzymes [6, 7, 8, 9]. Therefore, the profile and levels of amino acids can determine the types of amines formed. Bioactive amines are relevant compounds for every living cell as they are required for growth and health. They can be divided into polyamines and biogenic amines. The polyamines (spermine and spermidine) are ubiquitous and widespread from bacteria to mammals. They play important role as growth promoters, in the maturation and maintenance of the intestinal tract, and in the modulation of the immune response. They also show antioxidant activity [10, 11, 12]. The biogenic amines are naturally present in plants where they are precursors of hormones and can protect the plant against predators. They are also inherent to some animal tissues where they play relevant roles, e. g., histamine in mast cells and basophiles. Biogenic amines are also relevant to human health because of their neuro- and vaso-activities. However, at high concentrations some amines can cause adverse effects to human health. High histamine levels are associated with histamine poisoning whereas high levels of tyramine can cause migraine and hypertensive crises [7]. Biogenic amines can be

produced and accumulate in high protein food and also those submitted to fermentation processes. Most of the amines are heat resistant, remaining in the food after heat treatment. Therefore, the determination of bioactive amines in foods is important as indexes of quality and safety [6, 7, 8, 9]. Furthermore, the profile and levels of amines can be valuable for authenticity purposes [13, 14].

Mushrooms have long been regarded as health-promoting food. They are low in energy (low fat) and are good sources of dietary fibers, minerals (K), vitamins (provitamin D2, vitamin B12) and essential amino acids [15, 16, 17, 18, 19, 20]. They also have functional properties, including antioxidant, antimicrobial, hypocholesterolemic, hypoglycaemic, immunomodulating, and anti-tumor effects [15, 21, 22]. Mushrooms are considered to be good sources of bioactive amines [23, 24] and amino acids [5, 17, 19, 25, 26].

Several methods are available for the determination of bioactive polyamines [27, 28, 29], bioactive amines [24] (Dadáková et al., 2009) and amino acids [5, 17, 19, 25, 26] in mushrooms. Various analytical methods have been developed for the simultaneous quantification of amino acids and biogenic amines in fermented food – wine, beer, cheese and sausage [8, 30, 31, 32, 33, 34]. The technique involves acid extraction, with or without pre-column derivation, separation and quantification by reverse phase HPLC, UHPLC and ultraviolet, fluorescent and mass spectrometry detection. However, to our knowledge, no method dealing with the simultaneous determination of amino acids and biogenic amines in mushrooms has been described in the peer review literature.

In this context, the simultaneous determination of bioactive amines and amino acid in mushrooms would be a useful tool to access and monitor the identity, quality (functional properties, taste), safety and potential for amine formation during processing and storage. In addition, the simultaneous analysis of bioactive amines and amino acids in food represents an advance in separation techniques [8].

The aim of this study was to optimize a green, rapid and reliable analytical method for the extraction of amines and amino acids from mushrooms using trichloroacetic acid (TCA). A Plackett-Burman design and a Central Composite Rotational Design (CCRD) were used for the optimization of extraction. The extracted analytes were derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) and separated and quantified by ultra-performance liquid chromatography and UV

detection (UHPLC-UV). The method was validated for the simultaneous analysis of a large number of free bioactive amines and amino acids in mushrooms for the first time and applied to real samples.

2. Material and methods

2.1. *Sample and reagents*

Agaricus bisporus mushrooms (fresh and canned) were purchased from distributors (Ceasa, Campinas, SP, Brazil) at three different times. The mushrooms were freeze dried at -40 °C for 72 h using a L101, Liobras freeze drier (São Carlos, SP, Brazil). It was ground and sieved through 20 mesh. The moisture contents of the fresh and freeze-dried mushrooms were determined according to AOAC [35].

Bioactive amines, L-amino acids and ammonium ion standards were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA): spermidine trihydrochloride, spermine tetrahydrochloride, agmatine sulfate, putrescine dihydrochloride, cadaverine dihydrochloride, histamine dihydrochloride, tryptamine, serotonin hydrochloride, tyramine hydrochloride, 2-phenylethylamine hydrochloride, ammonium chloride, alanine, arginine hydrochloride, aspartic acid, cystine, glycine, glutamic acid, glutamine, histidine hydrochloride, isoleucine, leucine, lysine hydrochloride, methionine, phenylalanine, proline, serine, threonine, tyrosine, valine, and norvaline.

The AccQ.Fluor™ pre-column derivatization kit (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC), borate buffer and acetonitrile) was purchased from Waters (Milford, MA, USA).

The reagents were of analytical grade, except acetonitrile, which was LC grade. Ultrapure water was obtained from Milli-Q™ (Millipore Corp., Milford, MA, USA). The organic and aqueous solvents for UHPLC analysis were filtered through 0.22 µm pore size HAWP and HVWP membranes, respectively (Millipore Corp., Milford, MA, USA).

2.2. Methods

2.2.1. Chromatographic conditions

A Waters AcquityTM Ultra Performance LC (UPLC) system (Waters, Milford, MA, USA) equipped with an AcquityTM tunable ultra violet (TUV) detector at 249 nm was used. The column was a CSH C18 (50 x 2.1 mm, 1.7 µm i.d., Acquity UPLC). The solvent system consisted of A – 0.01 mol/L sodium acetate adjusted to pH 4.80 with acetic acid and B – acetonitrile, at gradient elution (flow rate of 0.9 mL/min) as follows: initial–2.5 min/0–0% B; 2.8–4.5 min/0–3% B; 4.5–10.0 min/3–30% B; 10.0–11.0 min/30–100% B; 11.0–11.75 min/100–100% B; 11.75–12.5 min/100–0% B, and further re-equilibration at initial conditions for another 2.5 min, thus indicating a total cycle time of 15 min until the next injection. Injection volume was set to 2 µL. The identification of the amines, ammonium ion and amino acids was performed by comparison of the retention time of the peaks of the analytes in the sample with those of the standard solution and also by adding the suspect analyte to the sample. The concentration of the analytes was calculated by interpolation in the respective analytical curves and the recovery of the internal standard (L-norvaline) was also used in the calculation.

2.2.2. Optimization of the derivatization procedure

The derivatization of the analytes according to Fiechter et al. [8] had to be optimized in special for spermine and spermidine. The amounts of AQC were increased until better recoveries were achieved.

2.2.3. Optimization of the extraction procedure

2.2.3.1. *Screening of variables affecting bioactive amines and amino acids extraction from mushrooms.*

A Plackett-Burman design was used to screen the main factors that could affect the recovery of amines and amino acids from mushrooms. The design included 12 tests and four repetitions at the central point for six independent variables (Table 1 and

Table 1A. - supplementary data). The selected independent variables were acid concentration, shaking time, shaking speed, centrifugation speed, centrifugation time and number of successive extractions.

Table 1. Experimental values and coded levels of the independent variables used in a Plackett-Burman design to screen conditions for the extraction of bioactive amines, amino acids and ammonium ion from mushrooms.

Independent variables	**Coded levels**		
	-1	**0**	**+1**
X_1 TCA* (%)	5	10	15
X_2 Shaker time (min)	2	4	6
X_3 Shaker speed (rpm)	300	550	800
X_4 Centrifugation speed (*g*)	7000	10500	14000
X_5 Centrifugation time (min)	4	8	12
X_6 Number of successive extractions	2	3	4

*TCA = Trichloroacetic acid.

Freeze dried mushrooms (200 µg) were incorporated with 800 µL of the 10 bioactive amines, ammonium ion and 20 amino acids standard solutions at a concentration of 2.5 mmol/L. The samples were homogenized thoroughly. 3 µL TCA were added to the sample and it was mixed in a shaker (Ovan, Barcelona, Spain). The mixture was centrifuged at 4 °C (Jouan MR23I, Saint Herblain, France). The supernatant was collected and filtered through a Whatman #1 filter paper.

Prior to derivatization, 40 µL of the internal standard L-norvaline (50 mmol/L) was added and the volume was brought up to 10 mL in a volumetric flask. Derivatization was undertaken under the conditions optimized for mushroom samples. An aliquot of the extract (500 µL) was neutralized using 300 µL of 0.1 mol/L NaOH. After homogenization, 5 µL of the neutralized extract was mixed with 30 µL of AccQ.Fluor® borate buffer and 15 µL of AQC. It was allowed to rest for 1 minute and then it was heated in a water bath at 55 °C for 10 minutes. The extract was filtered using a PTFE 0.22 µm pore size membrane (Minisart SRP 4®, Sartorius, Gottingen, Germany) and analyzed by UHPLC. The results were reported as percent recoveries.

2.2.3.2. *Optimization of the extraction.*

Based on the Plackett-Burman results, a Central Composite Rotational Design (CCRD) was conducted with 14 tests and three replications at the central point to optimize the recoveries during extraction of amines and amino acids. The independent variables considered were the acid concentration, shaker speed and number of successive extractions. The shaker time was set to 2 min, centrifugation time and speed were set to 4 min and 7,000 *g*, respectively. The experimental values and coded levels for the independent variables used in the CCRD are indicated in Table 2 and Table 2A - supplementary data. According to rotatable conditions [$\alpha = (2^3)^{1/4}$] for CCRD, α should be -1.68 and + 1.68, but they were set at -2 and +2 for number of successive extraction once this is a discrete variable. For the acid concentration, -α was equal to -1.3, which corresponded to 1.6% of TCA, once the concentration of TCA solution at -1.68 level would be bellow to zero. The statistically significant experimental results from the CCRD provided optimized extraction conditions which were used for method validation.

Table 2. Experimental values and coded levels of the independent variables used in a Central Composite Rotatable design (CCRD) to optimize conditions for the extraction of amino acids, bioactive amines and ammonium ion from mushrooms.

Independent variable	**Coded levels**				
	-1.68	**-1**	**0**	**+1**	**+1.68**
X_1 TCA* (%)	1.6	4	12	20	26
X_2 Shaker speed (rpm)	196	360	600	840	1000
X_3 Number of successive extractions	1	2	3	4	5

*TCA = Trichloroacetic acid. α ¸ is different from −1.68 for X1 and from ±1.68 for X3.

2.2.4. Validation of the method

The fitness of the optimized method for the analysis of 18 amino acids, 10 amines and the ammonium ion in mushrooms was evaluated according to the Commission Decision 2002/657/EC [1]. The following parameters were determined: specificity, calibration curves, accuracy, precision, and limits of detection and quantification.

2.2.4.1. Specificity.

Twenty different samples of mushroom were analyzed to evaluate the specificity of the method. The existence of any interference (possible peaks) that could affect detection in the range of retention times of the target analytes was investigated. The samples were spiked with the 10 amines, ammonium ion and 18 amino acid standards solutions and analyzed. Co-chromatography was employed to certify the identification of the peaks; the samples were spiked with each single standard to confirm the increase of the signal of the suspect peak to the respective standard added.

2.2.4.2. Calibration curves.

The existence of matrix effect was assumed once mushroom is a natural source of free amino acids [5, 17] and bioactive amines [24]. The calibration curves were constructed in solvent and in the mushroom extract - matrix. Calibration curves in solvent were constructed by spiking a 0.1 mol/L HCl solution with seven concentrations of all amines, ammonium ion and amino acids (2, 10, 20, 30, 40, 50 and 60 pmol on column, respectively) and five replicates at each level. The matrix calibration curves were constructed at the same concentration levels using 40 mg of freeze-dried mushrooms extracts instead of the solvent. Five replicates of each level were used. The samples were derivatized and injected randomly.

For each analyte, a plot of the analyte signal and internal standard (L-norvaline) signal ratio (SA/SI) versus concentration was built and the linear equation and the correlation coefficient were calculated by least squares linear regression.

2.2.4.3. Accuracy and precision.

Known levels of the analytes were added to eighteen aliquots of 40 mg of freeze-dried mushrooms to determine accuracy and precision (repeatability and reproducibility). These aliquots were divided into three groups of six replicates and they were fortified in the beginning of the extraction with 20, 40 and 60 pmol on column levels, respectively, of the amines, ammonium ion and amino acids standard solutions. The samples were analyzed and the concentrations for each aliquot and for each level

were calculated. Then, accuracy was calculated as [100*mean concentration found/fortification level] [1]:

Repeatability was established through evaluation of the coefficient of variation for each level (20, 40 and 60 pmols on column levels). The analyses were carried out on three different days by different analysts in order to evaluate reproducibility. Mean concentrations, standard deviations and coefficients of variation were calculated for the fortified samples from each analyst [1].

2.2.4.4. *Limits of detection (LOD) and quantification (LOQ).*

The limit of detection was determined as the lowest concentration of the analyte corresponding to three times the signal-to-noise ratio. The limit of quantification was the lowest concentration of the analyte that could be determined with acceptable accuracy and precision. It was considered the first analyte concentration in the calibration curve [1].

2.2.5. Analysis of real samples

Three different lots of fresh and canned mushrooms (*Agaricus bisporus*) were purchased directly from distributors in the market of Campinas, São Paulo, Brazil. The samples were analyzed in triplicate (n = 9) for amino acids, bioactive amines and ammonium ion.

2.2.6. Statistical analysis

The Protimiza Experimental Design software (Campinas, SP, Brazil) was used to evaluate the Plackett-Burman and Central Composite Rotational designs (CCRD) at 10 and 5% of probability, respectively. The Past 3.19 software (UIO, Oslo, Norway) was used to evaluate the matrix curves fitness (Ordinal least squares regression, visual inspection of residues, Dublin-Watson and Breusch-Pagan tests at 5% probability). Normality and significance tests in real samples were evaluated at the same software (Shapiro-Wilk normality test, one-way ANOVA followed by Tukey's test at 5% probability).

3. Results and discussion

3.1. Screening of variables affecting bioactive amines, amino acids and ammonium ion from mushrooms

The recoveries of amino acids, amines and ammonium ion in the Plackett-Burman design varied from 48.2% for alanine to 131.7% for the ammonium ion (Table 3A - supplementary data). At the central points, satisfactory recoveries (80 to 110%) were obtained for most of the analytes, although the recoveries for alanine and agmatine were >110%, and those for serotonin and phenylethylamine were <80%. The probability values (*p*-value) of six independent variables are indicated in Table 3. The significance level of 10% was used to minimize the risk of excluding an important factor from the procedure in the following step. Among independent variables investigated, shaker speed (X_3) significantly affected the recoveries of alanine, leucine, valine, agmatine, histamine, and spermidine; the number of successive extractions (X_6) significantly affected the recoveries of serotonin and tryptamine; and the acid concentration (X_1) significantly affected the recoveries of cadaverine and glutamic acid. The other independent variables investigated, e.g. shaker time (X_2), centrifugation speed (X_4) and time (X_5) did not affect significantly the recovery of amino acids, amines and ammonium ion. Hence, the optimization of the extraction procedure was conducted based on variables that showed significant effects: shaker speed, number of successive extractions and acid concentration. Centrifugation time and speed were set at 4 min and 7,000 *g*, respectively, and the shaker time was set at 2 min.

3.2. Optimization of the extraction

The percent recoveries of the amino acids, amines and ammonium ion obtained by using the CCRD are indicated in Table 4. Adequate recoveries were achieved for most of the experimental conditions. The coefficients of variation (CV) from the three replicates at the central point for all the responses were also adequate, ranging from 1.8% for serotonin to 14.7% for histamine, which is consistent with the limits established by Commission Decision 2002/657/EC [1].

Table 3. Significant probability (p-value) of the effect of the independent variables X_1 (TCA, %); X_2 (shaker time, min); X_3 (shaker speed, rpm); X_4 (centrifugation speed, *g*); X_5 (centrifugation time, min); and X_6 (number of successive extractions) for the extraction of amino acids, bioactive amines and ammonium ion from mushrooms.

Class/Analyte	Probability / independent variables*					
	X_1	X_2	X_3	X_4	X_5	X_6
Amino acids						
Alanine	0.98	0.53	0.15	0.35	0.77	0.80
Arginine	0.28	0.88	0.20	0.64	0.59	0.41
Aspartic acid	0.59	0.82	0.41	0.35	0.61	0.98
Cystine	0.19	0.73	0.33	0.71	0.41	0.21
Glutamic acid	***0.07***	0.54	0.24	0.14	0.95	0.20
Glycine	0.43	0.90	0.51	0.65	0.87	0.74
Histidine	0.39	0.49	0.48	0.42	0.19	0.70
Isoleucine	0.47	0.67	0.13	0.76	0.53	0.66
Leucine	0.44	0.64	***0.10***	0.73	0.58	0.62
Lisyne	0.24	0.91	0.18	0.92	0.44	0.44
Methionine	0.18	0.90	0.20	0.77	0.29	0.28
Phenylalanine	0.35	0.85	0.13	0.99	0.43	0.45
Proline	0.85	0.66	0.17	0.28	0.56	0.68
Serine	0.43	0.17	0.87	0.12	0.94	0.98
Threonine	0.29	0.80	0.12	0.60	0.62	0.51
Tyrosine	0.31	0.73	0.13	0.85	0.43	0.55
Valine	0.38	0.61	***0.10***	0.64	0.57	0.65
Bioactive amines						
Agmatine	0.16	0.26	***0.04***	0.30	0.44	0.39
Cadaverine	***0.08***	0.94	0.12	0.64	0.62	0.19
Histamine	0.80	0.37	***0.06***	0.47	0.19	0.89
Phenylethylamine	0.17	0.95	0.16	0.76	0.58	0.21
Putrescine	0.27	0.72	0.29	0.95	0.33	0.51
Serotonin	0.57	0.51	0.37	0.46	0.36	***0.10***
Spermidine	0.17	0.47	***0.09***	0.70	0.59	0.37
Spermine	0.19	0.77	0.20	0.87	0.25	0.38
Tryptamine	0.24	0.84	0.24	0.76	0.86	***0.10***
Tyramine	0.21	0.99	0.23	0.83	0.53	0.33
Ammonium ion	0.69	0.97	0.80	0.66	0.30	0.67

*Values in bold and italic are significant ($p \leq 0.10$).

Table 4. Recovery of amino acids, bioactive amines and ammonium ion during extraction of amino acids, bioactive amines and ammonium ion from mushrooms using a Central Composite Rotatable design.

Class/Analyte	Recovery (%) / Design points																
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
Amino acids																	
Alanine	117.4	101.0	98.3	90.4	92.2	85.0	113.0	94.9	91.8	96.9	83.3	96.8	57.3	93.0	62.5	72.5	91.9
Arginine	99.5	93.2	90.8	92.0	91.6	85.5	97.8	89.3	89.7	93.7	87.6	95.5	73.9	90.7	59.8	61.1	92.7
Aspartic acid	99,6	67,3	92,3	90,0	71,3	57,3	97,9	70,1	96,4	69,5	88,0	71,5	58,3	97,4	66,5	67,1	70,2
Cystine	92.3	83.7	85.3	85.2	86.0	80.1	91.2	82.6	82.1	83.2	79.4	86.0	67.8	81.2	70.7	76.5	83.5
Glutamic acid	125,2	100,3	117,5	109,4	92,7	91,5	119,5	88,8	117,4	89,3	93,9	93,0	77,1	94,8	87,5	88,0	87,7
Glycine	101,9	97,3	91,5	88,4	59,1	86,8	99,8	86,5	90,3	91,8	85,8	94,9	71,1	90,1	84,0	83,5	89,1
Histidine	99.1	91.2	90.7	89.0	89.7	83.5	95.7	86.6	89.5	86.9	83.3	89.5	70.6	86.0	79.1	86.9	88.0
Isoleucine	98.0	91.1	89.4	90.4	89.6	83.6	96.5	87.4	88.0	88.4	82.8	90.6	70.0	85.7	73.7	75.3	87.7
Leucine	98.3	91.5	89.3	90.1	89.1	83.2	96.8	87.2	87.8	88.1	82.4	90.4	68.7	85.6	78.1	80.2	87.4
Lysine	95.2	89.5	87.7	88.3	87.7	81.7	93.9	85.2	85.9	85.8	81.1	88.1	69.4	83.4	77.5	80.3	85.5
Methionine	87.7	81.5	80.7	81.2	81.0	75.6	86.5	78.6	78.8	79.3	75.2	81.7	64.5	77.4	76.4	78.7	79.3
Phenylalanine	125.1	111.4	114.4	114.0	114.7	102.5	122.0	109.6	111.8	111.9	106.4	115.3	90.5	109.1	64.5	66.7	111.3
Proline	104.0	87.4	89.5	91.3	86.7	77.2	101.1	89.1	88.5	91.9	81.6	92.2	62.5	89.0	103.2	105.6	90.6
Serine	107.4	83.7	99.4	98.4	68.2	76.1	104.4	50.1	100.4	113.6	70.0	114.6	48.4	58.1	112.4	106.9	108.1
Threonine	102.8	98.5	93.4	97.0	92.2	89.2	100.5	94.7	91.9	92.0	85.8	94.1	72.1	89.2	81.0	88.2	94.8
Tyrosine	92.2	83.6	84.4	83.1	82.2	78.6	91.4	81.7	80.7	83.4	78.7	85.8	64.5	81.5	91.0	92.5	83.5
Valine	98.0	90.1	89.1	88.8	87.9	82.5	96.5	86.6	86.6	87.9	82.2	90.2	68.0	85.5	78.0	79.5	87.4
Bioactive amines																	
Agmatine	103.9	90.1	93.2	96.5	96.3	84.9	99.9	91.7	91.6	93.6	91.8	98.4	78.1	94.6	81.7	83.8	97.6
Cadaverine	98.9	88.5	89.6	90.6	92.9	82.2	96.9	88.4	89.2	88.0	83.0	90.9	71.8	85.5	79.1	81.7	88.0
Histamine	96.7	90.2	88.3	89.2	88.5	82.9	95.1	86.8	87.6	87.9	82.4	89.7	69.9	85.8	80.0	82.6	87.6
Phenylethylamine	78.6	71.3	71.3	73.1	74.1	66.4	77.9	70.5	70.9	70.4	67.0	73.7	55.5	68.4	82.4	84.0	71.3
Putrescine	96.3	89.5	88.6	90.7	90.8	83.3	94.9	87.3	87.8	88.1	83.2	90.3	72.6	85.4	66.1	67.5	88.0
Serotonin	72.6	65.1	68.3	66.1	71.2	61.1	73.2	65.4	70.0	66.5	60.9	66.7	55.6	66.7	86.9	89.9	68.6
Spermidine	103.2	93.9	92.6	98.4	100.0	87.8	103.7	95.0	95.1	94.7	89.6	98.9	76.9	91.2	108.9	114.2	96.1
Spermine	120.0	108.3	104.3	121.8	123.1	105.1	127.2	119.4	117.4	116.7	109.6	124.9	92.4	110.0	78.2	79.0	121.3
Tyramine	92.1	85.0	84.7	86.2	86.6	78.6	91.0	83.1	82.6	83.7	78.3	86.1	67.5	81.5	84.0	86.2	82.9
Tryptamine	93.5	82.5	86.2	86.1	89.9	79.0	93.9	85.2	87.6	84.3	80.7	89.3	69.2	84.9	79.4	95.2	88.9
Ammonium ion	121.3	109.8	103.4	104.4	107.1	87.3	102.5	105.8	97.3	102.6	96.2	103.4	73.6	99.7	75.8	90.8	102.2

Design points are indicated in Table 1A – at supplementary data.

The recoveries obtained for each amino acid, amine, and ammonium ion were near the central point's results; therefore, it was not possible to build models for the extraction of these analytes [36]. These results indicate that the variables used - number of successive extractions, TCA concentration and shaker speed were not statistically significant for analyte recovery. No linear, quadratic or interaction effects between the variables were significant. However, it is important to observe that when the number of successive extractions was equal to one, the recovery was not satisfactory. Therefore, the optimum condition for the extraction of amino acids, amines and ammonium ion from mushrooms were 2 successive extractions with 2 min shaking at 200 rpm and 4 min centrifugation at 7,000 *g* using 1.6% TCA.

3.3. Chromatographic and derivatization conditions

The chromatographic conditions of the method developed by Fiechter et al. [8] for the separation and quantification of amino acids and amines in cheese were not adequate for our "in house" conditions. Considering the column used Waters® CHS C18, 50 x 2.1 mm, 1.7 µm i.d. column and the health and care recommendations from the manufacturer [37], the UHPLC conditions were established as 0.01 mol/L sodium acetate in ultrapure water adjusted to pH 4.80 with acetic acid and HPLC grade acetonitrile as mobile phases, 35 ˚C column temperature and a gradient elution operated at a flow rate of 0.9 mL/min (backpressure <690 Bar).

In the derivatization of amino acids, amines and ammonium ion using AQC-tag Waters®, the proportions of the derivatizing reagents [8] had to be changed. Better performances and linearity, especially for spermidine and spermine, were obtained by using 5 µL of the neutralized sample extract, 30 µL of borate buffer and 15 µL of AQC-tag.

3.4. Validation of the method

3.4.1. Specificity and selectivity

The chromatographic conditions were optimized to provide the shortest possible run of all analytes of interest with appropriate resolution. The chromatographic run had a total time of 15 min and all analytes eluted within 12 min. The shortest retention time was observed for aspartic acid (4.75–4.81 min), which had the highest affinity to the mobile phase – acetonitrile and lowest interaction with the stationary phase. On the other hand, the longest

retention time was observed for spermine (10.9–11.0 min). The method was not selective for glutamine in mushrooms, however for the others amino acids, as indicated in the chromatograms (Figure 1), the proposed sample preparation procedure and UHPLC conditions provided peaks with appropriated resolution, attesting the specificity and selectivity of method.

3.4.2. Analytical curves

Since some amino acids and amines are naturally present in mushrooms [5,17,24] the matrix effect of the samples was assumed. The linear equations for the analytical curves, the determination coefficients (R^2) and the range of concentration of the amino acids, amines and ammonium ion in the matrix are indicated in Table 5. The data fitted a linear regression model with R^2 equal or above 0.96. Thereby, adequate linearity within the working range for all analytes was achieved.

3.4.3. Limits of detection (LOD) and quantification (LOQ)

The results for the LOD and LOQ for each amino acid, bioactive amine, and ammonium ion are indicated in Table 5. The limits of detection were established as the lowest concentration of the analyte corresponding to three times the signal-to-noise ratio and varied from 0.04 to 0.58 mg/100 g, the lowest for the ammonium ion and the highest for cystine. The limits of quantification were established as the first points of calibration curves and varied from 0.14 to 1.92 mg/100 g. When comparing the limits of quantification of some amines with those in the literature [27], lower LOQ were found for spermidine, spermine and putrescine. These values are adequate for the analysis of these compounds in mushroom.

3.4.4. Accuracy, repeatability, reproducibility

The concentrations of the analytes in the samples spiked at three different levels, the coefficients of variation of repeatability and reproducibility and accuracy are indicated in Table 6. According to the Commission Decision 2002/657/EC [1], when the analyte concentration is higher than 10 µg/kg, the acceptable range of recovery must be between 80% and 110%. The mean recoveries for most of the studied analytes attended this criterion; however, a few analytes were out of this range, e.g. phenylethylamine and serotonin (~70%). The maximum CV for repeatability must be between 1/2 and 2/3 of the

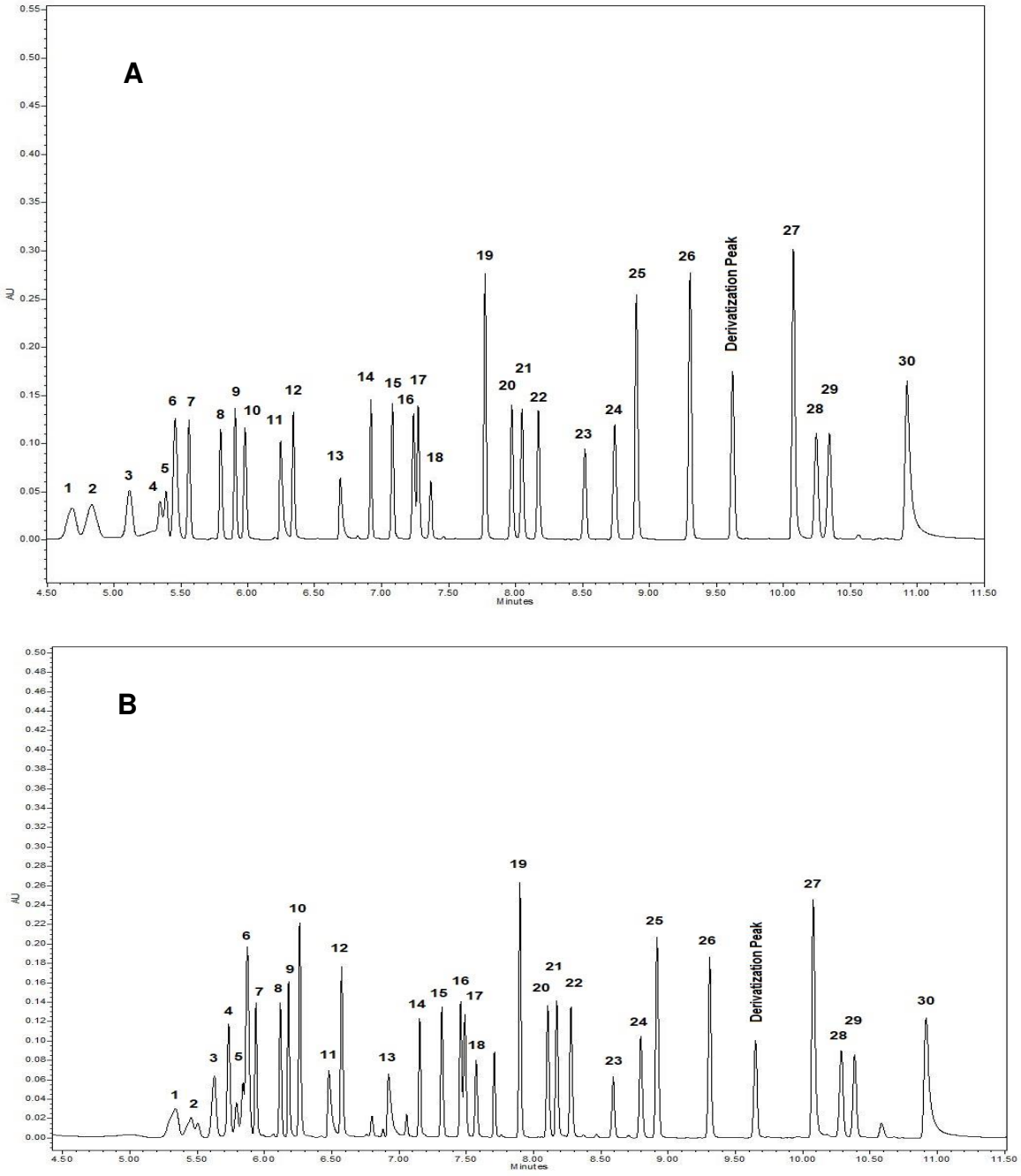

Figure 1. UHPLC separation of AQC derivatized standard solution containing 18 amino acids, 10 bioactive amines and ammonium ion in solvent (A) and in mushroom extract (B) using CSH C18 column (50 x 2.1 mm, 1.7 µm i.d., Acquity UPLC), gradient elution of A 0.01 mol/L sodium acetate, pH 4.80 and B – acetonitrile and UV detection at 249 nm. Legend: 1 - aspartic acid, 2 - serine, 3 - glycine, 4 - glutamic acid, 5 - glutamine, 6 - ammonium ion, 7- histidine , 8 - threonine, 9 - arginine, 10 - alanine, 11 - histamine, 12 - proline, 13 - agmatine, 14 - cystine, 15 - tyrosine, 16 - valine, 17 - methionine, 18 - norvaline (Internal standard), 19 - lysine, 20 - isoleucine, 21 - leucine, 22 - phenylalanine, 23 - serotonin, 24 - tyramine, 25 - putrescine, 26 - cadaverine, 27 - spermidine, 28 - 2-phenylethylamine, 29 – tryptamine, and 30 -= spermine.

Table 5. Limits of detection (LOD) and quantification (LOQ), range of concentrations in the calibration curves and linear equations of analytical curves in the matrix of the method for the analysis of amino acid, bioactive amines and ammonium ion in mushrooms by UHPLC-UV.

Class/Analyte	Limits (mg/100 g)		Range calibration curves (mg/100 g)	Linear equations (R^2)
	LOD	LOQ		
Amino acids				
Alanine	0.21	0.71	0.71 – 21.38	y = 0.0264x + 1,4707 (0.971)
Arginine	0.42	1.39	1.39 – 41.81	y = 0.0321x + 0,1801 (0.990)
Aspartic acid	0.32	1.06	1.06 – 31.94	y = 0.0268x + 0.1984 (0.989)
Glutamic acid	0.35	1.18	1.18 – 35.3	y = 0.02756 + 0.5409 (0.996)
Cystine	0.58	1.92	1.92 – 57.67	y = 0.0292x + 0.0096 (0.997)
Glycine	0.18	0.60	0.60 – 18.02	y = 0.0266x + 0.3829 (0.957)
Histidine	0.28	0.92	0.92 – 27. 64	y = 0.0321x + 0.0964 (0.992)
Isoleucine	0.31	1.05	1.05 – 31.48	y = 0.0351x + 0.1971 (0.991)
Leucine	0.31	1.05	1.05 – 31.48	y = 0.0361x + 0.2761 (0.987)
Lysine	0.35	1.17	1.17 – 35.09	y = 0.0613x + 0.1975 (0.993)
Metionine	0.36	1.19	1.19 – 35.81	y = 0.0357x + 0.0601 (0.995)
Phenylalanine	0.40	1.32	1.32 – 39.65	y = 0.0372x + 0.1656 (0.989)
Proline	0.28	0.92	0.92 – 27.63	y = 0.0331x + 0.8698 (0.987)
Serine	0.25	0.84	0.84 – 25.22	y = 0.0178x + 0.0832 (0.988)
Treonine	0.29	0.95	0.95 – 28.59	y = 0.0292x + 0.2351 (0.987)
Tyrosine	0.43	1.45	1.45 – 43.49	y = 0.0351x + 0.1341 (0.996)
Valine	0.28	0.94	0.94 – 28.12	y = 0.0336x + 0.2474 (0.990)
Bioactive amines				
Agmatine	0.31	1.04	1.04 – 31.25	y = 0.0292x + 0.0024 (0.996)
Cadaverine	0.25	0.82	0.82 – 24.52	y = 0.0639x + 0.0905 (0.994)
Histamine	0.27	0.89	0.89 – 26.68	y = 0.0231x + 0.0337 (0.981)
Phenylethylamine	0.29	0.97	0.97 – 29.08	y = 0.0394x + 0.0452 (0.993)
Putrescine	0.21	0.71	0.71 – 21.16	y = 0.0666x + 0.0667 (0.994)
Serotonin	0.42	1.41	1.41 – 42.29	y = 0.0258x – 0.0845 (0.994)
Spermidine	0.35	1.16	1.16 – 34.86	y = 0.0837x + 0.2534 (0.987)
Spermine	0.49	1.62	1.62 – 48.56	y = 0.0814x - 0.1845 (0.991)
Tryptamine	0.38	1.28	1.28 – 38.45	y = 0.0372x - 0.0163 (0.995)
Tyramine	0.33	1.10	1.10 – 32.92	y = 0.0365x + 0.0335 (0.994)
Ammonium ion	0.04	0.14	0.14 – 4.08	y = 0.0506x + 0.9772 (0.989)

y = peak area/internal standard peak area (25 pmol), x = analyte concentration in pmol. R^2 = determination coefficients.

Table 6. Mean concentration, coefficients of variation of repeatability (CVr) and reproducibility (CVR) and accuracy obtained after spiking mushroom samples with three different levels of amino acids, bioactive amines and ammonium ion during analysis by UHPLC-UV.

Class/Analyte	Spiking level (mg/100 g)	Mean level ± sd (mg/100 g)	Precision (%)		Accuracy (%)
			CV_r	CV_R	
Amino acids					
Alanine	7.1	8.2 ± 0.6	6.9	14.4	115.6
	14.3	16.0 ± 0.7	8.6	10.0	111.9
	21.4	21.0 ± 1.2	5.4	8.3	98.0
Arginine	13.9	11.8 ± 0.6	5.1	10.2	84.5
	27.9	25.3 ± 1.8	2.9	4.9	90.7
	41.8	34.1 ± 1.3	3.7	5.5	81.7
Aspartic acid	10.6	9.3 ± 0.7	7.4	14.6	87.6
	21.1	19.6 ± 1.1	5.4	14.2	93.1
	31.7	27.9 ± 1,4	5.2	9.4	87.9
Cystine	19.2	15.7 ± 0.8	4.8	8.0	81.7
	38.4	32.7 ± 2.1	4.4	4.4	85.0
	57.7	44.9 ± 1.8	4.0	5.1	77.9
Glutamic acid	11.8	10.9 ± 0.7	6.3	10.2	92.5
	23.5	21.1 ± 0.8	4.0	15.4	89.8
	35.3	25.7 ± 1.9	7.2	7.5	72.7
Glycine	6.0	6.4 ± 0.4	6.4	17.2	106.0
	12.0	8.7 ± 0.6	7.3	14.8	72.6
	18.0	18.9 ± 2.5	13.2	14.5	105.1
Histidine	9.2	7.8 ± 0.5	6.2	9.8	84.8
	18.4	16.6 ± 1.1	3.1	4.9	90.2
	27.6	22.2 ± 1.0	3.9	5.7	80.1
Isoleucine	10.5	8.8 ± 0.5	5.8	9.8	83.7
	21.0	18.7 ± 1.3	3.0	5.0	89.1
	31.5	25.4 ± 1.0	3.8	5.5	80.6
Leucine	10.5	8.8 ± 0.6	6.3	10.6	84.0
	21.0	18.8 ± 1.4	3.0	5.1	89.4
	31.5	25.1 ± 1.0	4.0	5.4	79.6
Lysine	11.7	9.5 ± 0.6	6.1	8.7	80.9
	23.4	20.2 ± 1.4	4.5	4.9	86.3
	35.1	27.7 ± 1.0	3.8	5.6	78.9
Methionine	11.9	8.7 ± 0.4	4.9	8.6	73.2
	23.9	19.4 ± 1.3	2.7	4.5	81.3
	35.8	26.8 ± 1.0	3.2	5.0	74.8
Phenylalanine	13.2	11.2 ± 0.5	4.5	9.0	84.8
	26.4	23.4 ± 1.5	3.0	4.5	88.5
	39.6	32.1 ± 1.2	3.8	5.5	81.1
Proline	9.2	9.0 ± 0.7	7.4	14.4	98.0
	18.4	18.7 ±1.0	6.7	10.4	101.3
	27.6	21.7 ± 0.5	5.1	6.1	78.7
Serine	8.4	8.4 ± 1.02	12.2	16.9	99.9
	12.0	10.8 ± 0.6	5.7	14.8	89.8
	18.0	12.8 ± 0.7	5.2	15.8	71.2

Table 6. Mean concentration, coefficients of variation of repeatability (CVr) and reproducibility (CVR) and accuracy obtained after spiking mushroom samples with three different levels of amino acids, bioactive amines and ammonium ion during analysis by UHPLC-UV **(continue).**

Class/Analyte	Spiking level (mg/100 g)	Mean level ± sd (n = 6) (mg/100 g)	Precision (%) CVr	CVR	Accuracy (%)
Threonine	9.5	8.2 ± 0.6	6.8	10.7	86.1
	19.1	17.8 ± 1.4	3.5	5.5	93.6
	28.6	23.5 ± 0.9	3.9	5.6	82.1
Tyrosine	14.5	11.3 ± 0.6	5.3	9.4	77,6
	29.0	24.6 ± 1.8	2.9	4.9	84.9
	43.5	33.6 ± 1.1	4.4	4.7	77,2
Valine	9.4	7.6 ± 0.5	6.3	10.5	80.8
	18.7	17.0 ± 1.1	3.2	5.0	90.7
	28.1	22.1 ± 0.9	4.2	6.0	78.5
Bioactive amines					
Agmatine	10.4	10.2 ± 0.7	7.3	10.2	97.7
	20.8	19.8 ± 1.2	2.4	4.4	95.2
	31.2	26.3 ± 1.1	4.1	6.7	84.2
Cadaverine	8.2	6.8 ± 0.1	1.9	8.5	83.6
	16.3	14.6 ± 1.0	2.8	4.9	89.5
	24.5	20.5 ± 0.9	3.9	5.3	83.6
Histamine	8.9	9.9 ± 0.2	2.4	10.2	111.7
	17.8	19.9 ± 0.7	2.7	4.4	111.8
	26.7	27.3 ± 1.0	3.6	6.1	102.5
Phenylethylamine	9.7	5.4 ± 0.1	4.5	11.8	55.4
	19.4	14.0 ± 1.0	3.0	4.8	72.2
	29.1	20.6 ± 0.6	3.8	4.8	70.7
Putrescine	7.1	5.9 ± 0.1	2.5	8.0	83.1
	14.1	12.4 ± 0.8	2.8	4.5	87.7
	21.2	17.4 ± 0.6	3.3	4.6	82.1
Serotonin	14.1	9.3 ± 0.2	1.6	13.4	65.9
	28.2	19.9 ± 1.2	2.2	14.7	70.7
	42.3	30.2 ± 0.8	4.6	13.4	71.4
Spermidine	11.6	10.1 ± 0.5	5.4	10.4	87.2
	23.2	21.9 ± 1.3	4.7	4.8	94.3
	34.9	30.7 ± 1.1	3.5	5.9	88.1
Spermine	16.2	17.0 ± 1.6	9.6	10.5	104.9
	32.4	35.8 ± 1.8	4.7	6.0	110.6
	48.6	54.0 ± 1.7	3.1	5.6	111.1
Tryptamine	12.8	10.4 ± 0.1	2.5	9.6	81.0
	25.6	22.3 ± 1.3	4.8	5.5	87.2
	38.5	32.3 ± 1.2	3.8	5.6	84.0
Tyramine	11.0	8.5 ± 0.3	3.5	7.9	77.1
	21.9	18.5 ± 1.3	2.7	4.9	84.3
	32.9	25.7 ± 0.9	3.3	5.8	77.9
Ammonium ion					
	1.4	1.3 ± 0.1	10.7	13.6	90.2
	2.7	2.8 ± 0.2	3.2	4.9	102.6
	4.1	3.6 ± 0.1	3.9	8.1	87.7

CV_r = repeatability - coefficient of variation; CV_R = reproducibility - coefficient of variation. Mean values were calculated using zero as nq (not quantified). Mean values with different letters in the same line are significantly different (Tukey test, $p \leq 0.05$)

CV for reproducibility. The repeatability for most of the analytes had a CV below 10%, with exception of the lowest spiking level for ammonium ion (10.7%) and for serine (12.2%). Concerning reproducibility, most of the analytes had a CV below 15%, except for the lowest spiking level of glycine (17.2%), and the lowest and highest spiking levels of serine (~16%). Based on these results, the method was considered fit for the analysis of amino acids, amines and ammonium ion in mushroom. Furthermore, this is the first multianalytes method for amino acids and amines in mushrooms.

3.5. Analysis of real mushroom samples

The validated method was used in the analysis of amino acids, bioactive amines and ammonium ion in fresh and canned Champignon (*Agaricus bisporus*) mushrooms (Figure 2). Among the ten bioactive amines investigated, only spermidine was detected in the samples and this polyamine was present at levels which varied from 6.6 to 8.9 mg/100 g in fresh and 4.3 and 7.7 mg/100 g in canned mushrooms. The levels of spermidine found in literature were between 4.4 mg/100 g [24] and – 19.2 mg/100 g [38] for *Agaricus bisporus*. In addition, these authors reported the presence of spermine and putrescine, along with spermidine in their studies, which were not detected in these samples.

When comparing the levels of spermidine in fresh and canned mushrooms, it seems like canned mushrooms can have lower levels compared to fresh ones. Further studies are needed to ascertain the role of canning on the polyamine content of mushrooms. Based on the polyamine – spermidine content found, *A. bisporus*, either canned or the great majority of mushrooms are classified as high (>1 mg/100 g) foods in polyamines. The presence of spermidine in mushrooms is relevant as this polyamine has several functional properties, including promotion of intestinal health, due to its role in the growth, maturation and regeneration of the mucosa [11, 39, 40], modulation of the immune system, and action as anti-inflammatory, antioxidant and cardioprotective agents [11, 39, 41, 42, 43].

Concerning the 17 amino acids analyzed, only methionine and cystine were not detected in fresh and canned mushrooms. In fresh mushrooms the average total level of amino acids was 460.3 mg/100 g. This result is similar to those

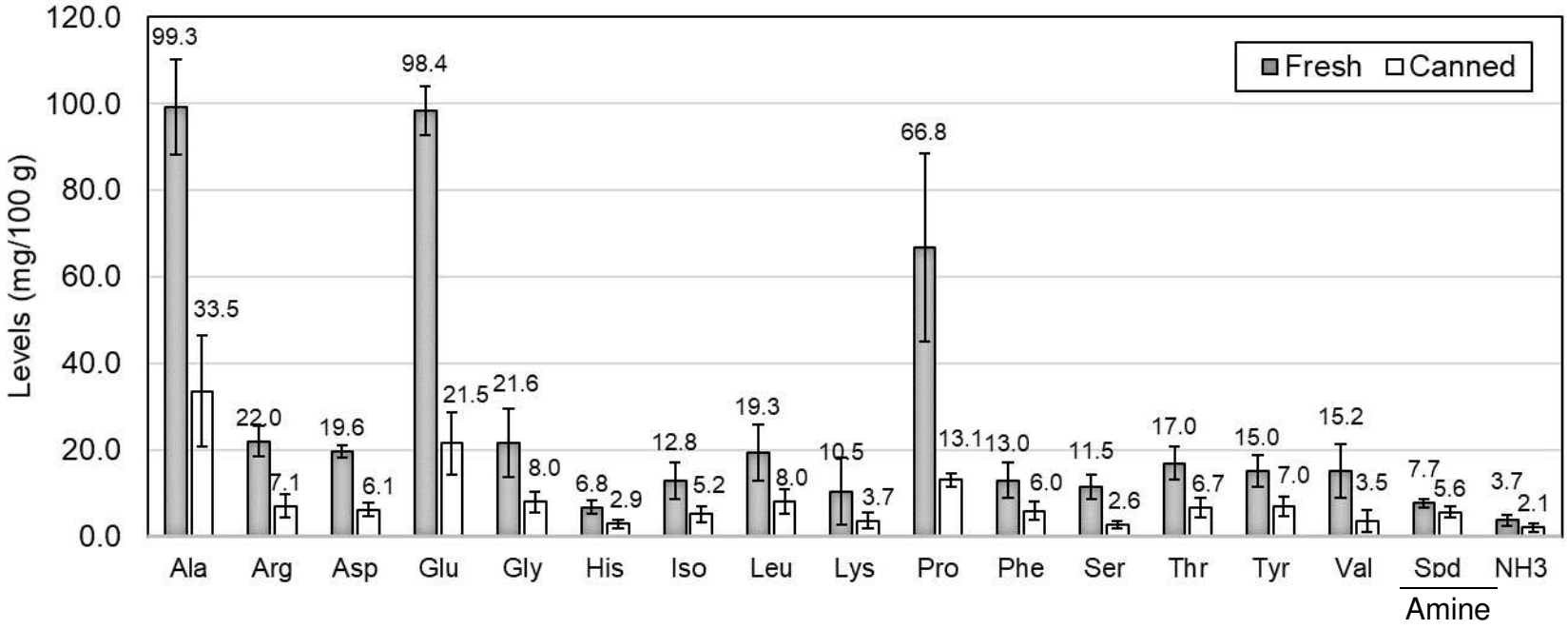

Figure 2. Levels of bioactive amines, amino acids and ammonium ions (mg/100g) of fresh and canned mushroom Champignon (*Agaricus bisporus*) from the market of Campinas, São Paulo, Brazil. Legend: Amino acids: Ala = alanine; Arg = arginine; Glu = glutamic acid; Gly= glycine; His = histidine; Iso = isoleucine; Leu = leucine; Lys = lysine; Pro = proline; Phe = phenylalanine; Ser = serine; Thr = threonine; Tyr = tyrosine; Val = valine. Bioactive amines: Spd = spermidine. NH_3 = ammonia

reported by Tsai et al. [44], Liu et al. [45], Pei et al. [46] but lower than total free amino acids levels found by Rotola-Pukkila et al. [26] for A. bisporus mushroom. Alanine (99.3 mg/100 g), glutamic acid (98.4 mg/100 g) and proline (66.8 mg/100 g) were the predominant amino acids with the highest contributions to the total levels, representing 21.6%, 21.4% and 14.5%, respectively. These results are similar to those reported by Liu et al. [45] and Rotola-Pukkila et al. [26]; however, in Tsai et al. [44] and Pei et al. [46] studies, histidine was the major amino acid found in *A. bisporus* mushroom.

In canned mushrooms, the average total level of amino acid was lower compared to fresh ones (144.3 mg/100 g) and similar to the total level found by Liu et al. [45]. As well as in the fresh mushrooms, alanine (33.5 mg/100 g), glutamic acid (21.5 mg/100 g) and proline (13.1 mg/100 g) were the amino acids present at the highest contents representing 23.2%, 14.9% and 9.1% of total levels. These results were higher for alanine and proline and lower for glutamic acid when compared to the results found by Liu et al. [45].

The presence of umami amino acids (glutamic and aspartic acid) in *A. bisporus* is reported in the literature [26, 44, 45, 46, 47]. The lower levels of these amino acids in the canned mushrooms are in accordance with literature data, suggesting high losses due to the process steps. For example, Liu et al. [45] found 16.8 mg/100 g of aspartic acid in fresh and 8.7 mg/100 g in canned (a loss of 48.2%) and 135.7 mg/100 g for glutamic acid in fresh and 40.2 mg/100 g in canned *A. bisporus* (a loss of 70.3%). Alanine and proline are classified like sweet tasted amino acid and glutamic acid is classified by its umami taste. These data were pertinent to the literature data, in which *A. bisporus* was characterized by the high content of alanine and glutamic acid [26, 45] (Liu et al., 2014; Rotola-Pukkila et al., 2019).

The presence of ammonium ion is common in mushrooms. This substance is produced by the composting of the substrate in the production of mushrooms as a result of the degradation of proteins of the compost. However, the ammonia extremely toxic to the mycelial growth of the *Agaricus* mushroom, since compounds with high moisture content may lead to increased ammonia concentration and the *Agaricus bisporus* mycelium may die or be inhibited by lower ammonium levels of human olfactory capacity, which is between 10 and 20

ppm [48] (Siqueira et al., 2011). This ion can also be used as a parameter to monitor protein hydrolysis.

4. Conclusion

An analytical method for the simultaneous determination of free amino acids, bioactive amines and ammonium ion by UHPLC with UV detection was optimized and validated using pre-column derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate. A new chromatography and derivatization conditions were established for ensuring adequate responses for most of the analytes and adequate column backpressure. Although it was not possible to estimate a mathematical model for extraction of bioactive amines and amino acids in mushrooms, it was possible to set fast and soft extraction conditions and optimize the sample preparation. The method was validated according to Commission Decision 2002/657/EC and most of the analytes had satisfactory accuracy, repeatability and reproducibility. When applied to the analysis of commercial mushrooms samples the method was fast, easy, precise and reliable. The analyzed samples of *A. bisporus* presented higher contents of spermidine, which is a relevant bioactive compound, and glutamic acid, which is an important non-volatile taste compound.

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Appendix A. Supplementary material

Table 1.A. Screening of the factors that affect the recovery of amino acids, bioactive amines and ammonium ion during extraction from mushrooms using a Plackett-Burman design with six independent variables.

Design points	Independent variables					
	TCA* (%)	Shaker time (min)	Shaker speed (rpm)	Centrifugation speed (*g*)	Centrifugation time (min)	Sucessive extractions
1	15	2	800	7000	4	2
2	15	6	300	14000	4	2
3	5	6	800	7000	12	2
4	15	2	800	14000	4	4
5	15	6	300	14000	12	2
6	15	6	800	7000	12	4
7	5	6	800	14000	4	4
8	5	2	800	14000	12	2
9	5	2	300	14000	12	4
10	15	2	300	7000	12	4
11	5	6	300	7000	4	4
12	5	2	300	7000	4	2
13	10	4	550	10500	8	3
14	10	4	550	10500	8	3
15	10	4	550	10500	8	3
16	10	4	550	10500	8	3

*TCA = Trichloroacetic acid.

Table 2.A. Screening of the factors that affect the recovery of bioactive amines, amino acids and ammonium ion during extraction from mushrooms using a Central Composite Rotatable design with three independent variables.

Design Points	Independent variable		
	TCA (%)	Shaker speed (rpm)	Sucessive extractions
1	4.0	360.0	2
2	20.0	360.0	2
3	4.0	840.0	2
4	20.0	840.0	2
5	4.0	360.0	4
6	20.0	360.0	4
7	4.0	840.0	4
8	20.0	840.0	4
9	1.6	600.0	3
10	25.5	600.0	3
11	12.0	196.4	3
12	12.0	1003.6	3
13	12.0	600.0	1
14	12.0	600.0	5
15	12.0	600.0	3
16	12.0	600.0	3
17	12.0	600.0	3

*TCA = Trichloroacetic acid.

Table 3.A. Recovery during extraction of amino acids, bioactive amines and ammonium ion from mushrooms using the Plackett-Burman design.*

Class/Analyte	Recovery (%)/Design points															
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Amino acids																
Alanine	84.9	94.3	126.3	85.9	48.2	93.0	79.8	85.3	63.4	87.3	77.3	63.1	120.8	111.3	119.4	122.7
Arginine	82.8	83.6	76.4	82.2	66.4	84.4	78.4	80.5	71.4	82.6	77.3	70.7	96.9	87.8	90.3	91.8
Apartic acid	63.2	95.0	88.9	93.9	56.3	68.2	81.4	90.8	73.2	67.0	77.7	65.4	78.3	91.3	72.2	104.1
Cystine	76.5	80.9	68.8	79.1	66.9	80.2	76.8	77.4	72.6	78.1	74.4	69.7	94.4	85.3	89.3	89.4
Glutamic acid	68.9	73.9	104.5	69.6	58.9	78.3	75.6	76.8	63.6	73.6	73.0	92.5	77.1	74.8	73.2	77.7
Glycine	78.4	82.1	79.7	82.7	63.8	85.7	73.6	79.7	64.9	91.2	71.7	66.9	71.1	118.4	111.3	120.0
Histidine	76.9	81.4	72.5	80.3	67.0	80.8	76.9	78.3	69.0	80.7	75.3	80.4	90.0	83.5	86.0	87.7
Isoleucine	78.5	83.6	78.3	81.2	68.4	83.4	78.9	82.1	70.4	78.9	78.7	72.3	97.0	87.2	89.4	91.2
Leucine	76.7	82.4	77.7	79.8	65.1	82.5	77.3	79.7	68.3	77.4	75.7	69.6	96.1	85.8	88.3	90.0
Lysine	77.5	82.1	73.2	80.1	67.3	81.6	77.5	79.5	69.5	79.0	76.2	70.4	93.7	84.5	87.1	88.2
Methionine	73.3	77.5	65.1	75.8	62.3	76.7	73.5	73.9	66.3	73.3	70.7	65.3	88.9	79.8	83.4	83.6
Phenylalanine	76.9	82.6	73.9	80.6	66.0	82.7	78.2	80.0	70.0	77.3	75.6	70.8	95.3	85.8	88.5	89.8
Proline	68.6	75.4	87.8	70.6	50.3	77.9	69.3	72.3	61.7	80.6	67.3	58.2	101.5	86.4	88.6	92.8
Serine	75.1	73.7	74.3	85.75	61.6	93.6	70.2	70.6	65.5	78.2	66.9	82.7	78.5	81.7	76.9	80.2
Threonine	78.7	83.8	78.5	81.4	66.5	83.8	78.5	80.6	69.5	81.7	76.8	71.0	94.9	86.8	89.4	91.3
Tyrosine	72.5	77.0	70.1	74.9	62.7	77.0	72.6	74.5	64.6	72.4	71.4	66.2	88.6	79.8	82.3	83.6
Valine	72.8	77.7	73.6	75.4	62.1	78.1	72.5	74.8	64.5	73.0	71.7	65.9	90.6	81.2	83.0	84.9
Bioactive amines																
Agmatine	101.4	106.9	96.9	104.3	89.2	106.7	100.2	104.0	93.1	83.7	99.8	92.4	124.8	113.0	116.9	118.5
Cadaverine	79.2	81.1	68.7	80.0	67.0	81.9	77.9	78.0	70.2	79.1	73.7	62.4	94.2	83.4	88.4	87.7
Histamine	89.4	96.0	82.7	93.1	77.4	96.2	89.6	89.3	80.7	78.8	85.5	69.4	108.8	96.2	102.9	101.3
Phenylethylamine	65.4	68.2	58.0	67.4	55.5	69.2	65.4	66.8	58.8	66.4	63.7	54.1	81.1	71.7	75.6	75.2
Putrescine	80.6	83.5	72.8	81.8	71.0	83.4	79.8	82.3	72.8	81.0	78.3	76.0	96.5	86.4	90.4	90.3
Serotonin	61.6	62.7	52.7	62.6	52.7	63.5	63.3	62.9	61.1	61.1	60.9	55.5	78.0	70.2	75.0	73.8
Spermidine	86.6	85.7	81.2	84.3	74.3	88.1	83.3	87.9	73.2	86.6	81.7	52.7	103.1	89.2	96.5	94.9
Spermine	90.2	76.5	79.5	78.9	75.7	85.1	80.0	90.0	71.0	98.1	78.7	31.5	105.2	84.0	101.3	93.4
Tryptamine	76.8	78.2	68.3	78.5	66.4	80.7	78.0	79.1	72.5	81.1	76.3	63.6	94.1	84.6	90.7	88.4
Tyramine	77.2	80.5	70.2	78.8	68.1	80.7	77.0	79.4	70.4	78.3	75.8	68.5	93.3	83.8	87.5	87.6
Ammonium ion $_3$	111.6	121.2	113.7	113.9	81.7	109.8	119.9	101.8	108.5	109.9	114.6	117.4	131.7	128.6	127.2	127.8

* Placket-Burman design as indicated in Table 1A.

CAPÍTULO III: Process effect on bioactive amines, free amino acids and ion ammonium in *Agaricus bisporus* mushrooms

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Abstract

The effect of cooking and canned process on the bioactive amine and amino acids in three different lots of *Agaricus bisporus* mushroom were assessed with a special focus on the polyamines content and biogenic amines precursors. A UHPLC method was used for the simultaneous determination of amino acids and biogenic amines in a single run in fresh and process mushrooms samples. After pre-column derivatization with 6-aminoquinolyl-N-hydroxy succinimidyl carbamate (AQC). Of ten bioactive amines, only spermidine was detected in the *Agaricus bisporus*. The spermidine content is maintained in the cooking process, but it decreases in the canned processing. Regarding the total amino acids, there was an average loss of 39.7% in the cooking process and 64.6% in the canned process. the high loss of amino acids in canned process will also lead to a greater loss in amino acids responsible for the flavor of that mushrooms. Principal Component Analysis and Hierarchical Cluster Analysis showed that the influence of the processing is greater than the influence of the lot in relation to the total content, since the processing by itself leads to a reduction in the amino acids content in the mushrooms.

Keywords: Spermidine, Glutamic Acid, MSG-like, Principal Component Analysis

1. Introduction

The world consumption of edible mushrooms has increased. According to the Food and Agriculture Organization of the United Nations (FAO), the mushroom crop production was 5.9 million tons in 2007 and increased to 10.2 million tons in 2012, presenting a growth of more than 73% in ten years (FAOSTATS, 2019). In Brazil, *Agaricus bisporus* is the most consumed mushroom being produced 8 thousand tons per year (ANPC, 2019).

Mushrooms are susceptible to biogenic amines formation and accumulation because of its high water activity, the presence of free amino acids (Tsai, 2008; Sun et al., 2017, Manninen et al., 2018; Rotola-Pukkila, 2019) and the favorable conditions for growth and production of amino acid decarboxylase enzymes by the microorganism. Besides that, it also presents a chemical composition favorable to undesirable changes by both endogenous and microbial enzymes leading to spoilage (Dadáková et al., 2009; EFSA, 2011; Kalac, 2013; Papageorgiou et al., 2018).

The *Agaricus bisporus* mushroom presents high spermidine contents (Reis, 2014, Kalač 2014). The presence of dietary polyamines (spermidine and spermine) can have various health benefits, including promotion of intestinal health, since polyamines are responsible for growth, maturation and regeneration of the intestinal mucosa (Kalač, 2014; Ramani, et al.,2014). In addition, polyamines can contribute to the development of the immune system and are effective in wound healing, are anti-inflammatory agents, have antioxidant activity and cardioprotective effects (Kalač, 2014; Ramani et al., 2014; De Cabo & Navas, 2016; Handa, et al., 2018; Sharma et al, 2018). However, polyamines should be avoided by individuals with cancer as they may increase tumor growth (Kalač, 2014; Ramani et al., 2014). Besides the spermidine, others bioactive amines can be detected like putrescine, tyramine, tryptamine and phenylethylamine (Dadáková et al., 2009).

In addition to spermidine, high levels of free amino acids have recently been found in mushrooms (Tsai, 2008; Sun et al., 2017, Manninen et al., 2018, Rotola-Pukkila, 2019). Amino acids are the most important building blocks of body tissues, enzymes and hormones, so they are indispensable for vital functions. When it comes to metabolic dysfunction, insufficient resorption, increased nutritional demand after

surgical trauma, and medical care of preterm infants and newborns, there is a beneficial evidence for amino acids targeted supplementation (Marchini et al., 2016). *Agaricus bisporus* are considered to be rich in umami substances (Li et al., 2011, Liu et al., 2014); which can mainly be attributed to the presence of glutamic and aspartic acids, known as umami amino acids or MSG-like amino acids. Other free amino acids are reported to have sweet (alanine, glycine, proline, serine, threonine), bitter (arginine, histidine, isoleucine, leucine, methionine, phenylalanine, tryptophan, valine) or neutral (lysine, tyrosine) taste characters (Tsai et al., 2007, Jaworska & Berna, 2013, Liu et al., 2014, Rotola-Pukkila et al., 2019). The nutritional and organoleptic properties of mushrooms can be altered by processing and storage conditions, which influence the mushrooms chemical composition (Liu et al., 2014). Thus, the effect of processing may cause changes in the profile and content of bioactive amines and amino acids in mushrooms. So, at the first time, the objective of this work was to evaluated the effect of cook and canned process on bioactive amines, amino acids and ion ammonium in *Agaricus bisporus* mushrooms.

2. Material and methods

2.1. Sample and reagents

Samples of *Agaricus bisporus* mushrooms were purchased from the Ceasa of Campinas city, SP. Five kilogram of mushroom was obtained three times in different weeks each. Bioactive amines and L-amino acids standards were purchased from Sigma Chemical Co. (St. Louis, MO, USA): spermidine trihydrochloride, spermine tetrahydrochloride, agmatine sulfate, putrescine dihydrochloride, cadaverine dihydrochloride, histamine dihydrochloride, tryptamine, serotonin hydrochloride, tyramine hydrochloride, 2-phenylethylamine hydrochloride, alanine, arginine hydrochloride, asparagine, aspartic acid, cystine, glycine, glutamic acid, glutamine, histidine hydrochloride, isoleucine, leucine, lysine hydrochloride, methionine, phenylalanine proline, serine, threonine, tyrosine, valine, and norvaline (internal standard). AccQ.FluorTM pre-column derivatization kit was purchased from Waters (Milford, MA, USA).

The reagents were of analytical grade, except HPLC solvent acetonitrile which was LC grade. Ultrapure water was from Milli-QTM (Millipore Corp., Milford, MA, USA). The organic and aqueous solvents for the HPLC analysis were filtered through 0.2 µm pore size HAWP and HVWP membranes, respectively (Millipore Corp., Milford, MA, USA).

2.2. Processing

Each mushroom lot was divided in three sub lots, discriminate by processing in fresh, cooked and canned mushrooms. The process steps are represented in **Figure 1**. Soller-Rivas et al. (2009) method was used to cook mushrooms. A hundred grams of fresh mushrooms were cooked in 1 L of boiling water (97º C) for 10 min. To prepare the canned mushrooms, technical recommendations for edible mushroom processing was followed (EMBRAPA, 2000). The mushrooms were washed and immersed in sodium bisulfite 1% for 2 min. After this step, the mushrooms were immersed in boiling water (97˚C) for 5 min. A hundred grams of mushrooms were added to 250 mL glass pots. The glass pots were completed with NaCl 3% and ascorbic acid 0.12% solution at 90˚C. The glass pots were sealed and pasteurized in boiling water bath (97˚C) until the cap level for 20 min. After this process, the glass pots sealed were turn over for 10 min and them cooled in ice bath until room temperature. The canned mushrooms were manteined to 7 days prior analysis. The moisture content of fresh, cooked and canned mushrooms was obtained (AOAC, 2012). Fresh, cooked and canned mushrooms were frozen dried at -40˚C for 72 hours using a (L101, Liobras, Sao Carlos, SP,), and them powder at 20 mesh.

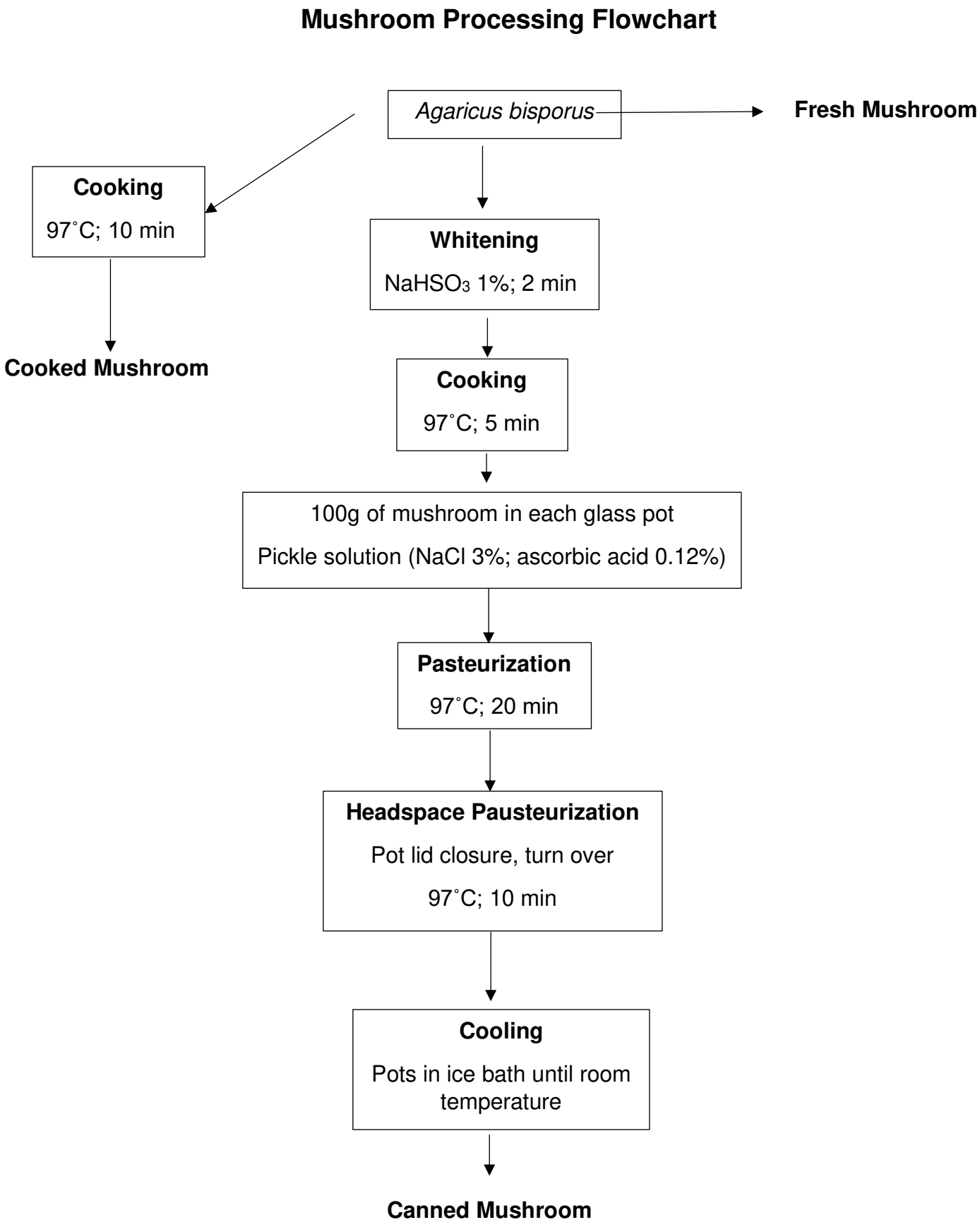


Figure 1. Mushroom processing flowchart.

2.3. Bioactive amines, amino acids and ammonium ions extraction from mushrooms

Bioactive amines, free amino acids and ion ammonium were extracted from 0.2 g for frozen dried fresh, cook and canned frozen mushrooms with 3 mL of 1.6% trichloroacetic acid. The samples were agitated for 2 min on a shaker at 200 rpm and centrifuged at 7,000 x g for 4 min at 4 °C. The extraction was repeated twice. The supernatant was collected and filtered through Whatman #1, 40 µL L-Norvaline, 50 mM (internal standard) was added them and bringing up the volume to 10 mL in a volumetric flask. An aliquot of the extract (500 µL) was neutralized using 300 µL of 0.1 mol/L sodium hydroxide. After homogenization, 5 µL of the extract was mixed with 30 µL of AccQ.Fluor® borate buffer and 15 µL AQC, it was allowed to rest for 1 minute and then, it was heated in a water bath at 55 °C for 10 minutes. The extract was filtered using PTFE 0.22 µm pore size membrane (Minisart SRP 4®, Sartorius, Goettingen, Germany) and analyzed by UPLC.

2.4. Chromatographic conditions

A Waters AcquityTM Ultra Performance LC (UPLC) system (Waters, Milford, MA, USA) equipped with an AcquityTM tunable ultraviolet (TUV) detector was used. The column was a CSH C18 (50 x 2.1 mm i.d., 1.7 µm, Acquity UPLC). The solvent system consisted of A – 0.01 mol/L sodium acetate adjusted to pH 4.80 with acetic acid and B – acetonitrile at gradient elution. Injection volume was set to 2 µL and the gradient elution was operated at a flow rate of 0.9 mL/min as follows: initial–2.5 min/0–0% B; 2.8–4.5 min/0–3% B; 4.5–10.0 min/3–30% B; 10.0–11.0 min/30–100% B; 11.0–11.75 min/100–100% B; 11.75–12.5 min/100–0% B, and further re-equilibration at initial conditions for another 2.5 min, thus indicating a total cycle time of 15 min until the next injection. Detection was possible using an ultra violet detection at 249 nm.

The identification of the bioactive amines, amino acids and ammonium ions was performed by comparison of the retention time of the analyte peaks in the sample with those of the standard solution and also by adding the suspect analyte to the sample. The concentration of bioactive amines, amino acids and ammonium ions was

calculated by interpolation in the respective analytical curves and the recovery of the internal standard were also used in the calculation.

2.5. Statistical analysis

The results were submitted to Shapiro Wilk test for normality and Levene's test to check homoscedasticity. Then, the data was submitted to analysis of variance and the means were compared by the Tukey test at 5% probability (Granato et al., 2014).

Two multivariate exploratory techniques, Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA), were applied for the characterization of mushrooms in relation to the profile and level of bioactive amines, amino acids and ion ammonium (Granato et al., 2018). All data were analysed using the Past 3.19 software (UIO, Oslo, Norway).

3. Results and Discussion

3.1. Process efect

3.1.1. Bioactive amines and amino acid content

For the first time the effect of processing on the mushroom *Agaricus bisporus* on the composition of bioactive amines and free amino acids was investigated. Mushrooms present high water content and water activity that affect the texture and contributes to the short shelf life of fruiting bodies (Kalač, 2009), moreover this characteristics and free amino acids content Beluhan & Ranogajec, 2011; Sun et al., 2017; Rotola-Pukkila, 2019), presenting favorable conditions for the formation of bioactive amines (Kalač & Křížek, 1996; Dadáková et al., 2009; EFSA, 2011). The composition of bioactive amines, free amino acids and ammonium ions are expressed on a wet basis, once they are consumed in this way, and are presented in **Table 1**. The harvest stage was not considered, since the mushrooms were purchased on the market at commercial maturity. Ten amines were analyzed, however only spermidine was detected in the *Agaricus bisporus*. This profile corresponds to the profile of this

species of mushroom (*Agaricus bisporus*) reported in a previous study by Reis et al. (2014). The occurrence of spermidine in mushroom, as well as in all living cells, has been reported in the literature (Okamoto et al., 1997; Dadaková et al., 2009; Kalač, 2013) and its presence is associated with diverse relevant roles in cellular metabolism and growth (Kalač, 2014; Ramani et al., 2014). The content of spermidine in the analyzed mushrooms varied from 5.0 mg/100g in canned mushrooms to 9.4 mg/100g in cooked mushrooms. This range levels classify these mushrooms as foods with a high content of polyamines (1 to 10 mg/100g) (Kalač, 2014). Regarding spermidine, for each lot cooking did not significantly affect the content of this polyamine on *Agaricus bisporus*. The processing for canned mushroom production led to a significant spermidine reduction, representing an average loss of 10.8%. The polyamines have in their structure free nitrogen groups. In the acid medium of the pickle (pH <4.5) this substance is expected to have increased solubility. Despite this loss, canned mushrooms still have high content of polyamines. Once dietary polyamines are essential for the maintenance of normal growth, maturation of the intestinal tract (Ali et al., 2011) and advantageous during the periods of wound healing, post-operational recovery, liver regeneration, or compensatory growth of the lung or the gut (Kalač, 2014).

The effect of heat treatment on the levels of tryptamine, phenylethylamine, putrescine, cadaverine, histamine and tyramine in the fresh, boiled and canned *Volvarela volvacea* (straw mushrooms) was evaluated by Yen (1992). In this study, the total amount of these six amines in fresh straw mushroom was reduced from 147.7 $mg.kg^{-1}$ to 28.1 $mg.kg^{-1}$ (wet basis) after cooking, a reduction of about 80%. This reduction can be attributed to the loss of water during cooking, since it includes the loss of histamine, tyramine and putrescine which have been reported to be thermostable. It can be seen that canned straw mushroom contained low concentrations of putrescine, 2-phenylethylamine and tyramine. As in the canned. process applied in Agaricus bisporus, the decrease on bioactive amines levels in canned straw mushroom might be due to loss in the blanching process before canning.

Kalač and Krizek (1997) evaluated the effect of storage on the content of putrescine and cadaverine in A. bisporus in intact fresh, sliced fresh, sliced cooked mushroom. When stored at 6°C for 48 hours, putrescine and cadaverine were not detected in these mushrooms. When stored at 20°C for 48 hours, it was quantified from

Table 1. Profile and levels (mg/100g wet basis) of bioactive amines, amino acids and ammonium ions in three lots of fresh, cooked and canned *Agaricus bisporus* mushroom.

	Fresh			Cooked			Canned		
Class/Analyte	Lot A (n =9)	Lot B (n = 9)	Lot C (n = 9)	Lot A (n = 9)	Lot B (n = 9)	Lot C (n = 9)	Lot A (n = 9)	Lot B (n = 9)	Lot C (n =9)
Amino acids									
Aspartic acid	20.1 ± 1.4 a	16.3 ± 2.9 a	17.8 ± 1.1 a	14.0 ± 4.1 b	9.0 ± 1.6 c	14.6 ± 2.2 b	9.6 ± 2.2 c	5.7 ± 2.0 c	7.1 ± 0.6 c
Alanine	105.7 ± 7.4 a	97.6 ± 4.9 a	91.6 ± 6.4 a	66.6 ± 10.1 b	40.3 ± 12.6 cd	66.6 ± 19.9 b	51.0 ± 14.7 bc	24.5 ± 5.3 d	47.1 ± 2.0 c
Arginine	25.1 ± 1.7 a	18.7 ± 0.8 bc	21.6 ± 1.7 ab	12.4 ± 5.1 de	8.9 ± 3.2 efg	16.0 ± 3.3 cd	10.5 ± 1.1 ef	5.0 ± 1.2 g	7.1 ± 0.8 fg
Glutamic acid	98.6 ± 7.2 a	94.2 ± 5.5 a	97.4 ± 4.8 a	56.9 ± 7.0 b	41.7 ± 6.4 bc	56.8 ± 20.5 b	33.0 ± 3.3 cd	21.3 ± 6.1 de	16.5 ± 3.6 e
Glycine	27.6 ± 4.2 a	20.0 ± 5.4 b	19.1 ± 5.8 b	22.0 ± 1.1 a	10.6 ± 4.2 c	11.4 ± 1.4 c	11.5 ± 3.7 c	6.3 ± 1.0 c	7.4 ± 0.5 c
Histidine	8.1 ± 0.6 a	5.0 ± 0.3 bc	7.5 ± 0.9 a	6.0 ± 1.1 b	2.8 ± 0.9 de	5.8 ± 0.5 b	4.1 ± 1.2 cd	2.3 ± 0.4 e	4.2 ± 0.3 cd
Isoleucine	16.2 ± 1.1 a	7.7 ± 0.4 c	14.6 ± 1.2 a	11.9 ± 2.5 b	4.7 ± 1.8 d	11.3 ± 1.1 b	7.6 ± 1.8 c	3.6 ± 0.7 d	7.0 ± 0.5 cd
Leucine	24.0 ± 1.7 a	11.3 ± 0.6 d	22.0 ± 1.7 ab	18.1 ± 4.0 bc	7.2 ± 2.8 e	17.2 ± 1.8 c	11.3 ± 2.7 d	5.5 ± 1.0 e	10.9 ± 0.7 de
Lysine	19.1 ± 1.3 a	2.8 ± 0.2 d	9.2 ±0.9 c	12.5 ± 1.9 b	3.8 ± 1.5 d	7.1 ± 1.8 c	6.9 ± 0.9 c	2.9 ± 0.7 d	3.2 ± 0.5 d
Phenylalanine	16.1 ± 1.1 a	8.0 ± 0.4 c	15.6 ± 2.0 a	12.8 ± 2.4 b	5.0 ± 2.0 d	11.3 ± 1.3 b	7.8 ± 1.5 c	4.2 ± 0.6 d	8.4 ± 0.4 c
Proline	38.4 ± 2.7 d	89.8 ± 3.7 a	66.5 ± 6.3 b	24.7 ± 2.2 ef	31.0 ± 9.7 de	49.0 ± 14.6 c	19.4 ± 5.3 f	16.7 ± 5.7 f	15.6 ± 4.0 f
Serine	13.0 ± 1.3 a	7.9 ± 0.5 c	11.6 ± 2.0 a	7.4 ± 1.3 c	4.1 ± 0.8 e	8.9 ± 2.2 b	5.3 ± 2.4 d	1.7 ± 2.0 f	2.0 ± 0.9 f
Threonine	19.9 ±1.4 a	12.4 ± 0.6 bc	18.8 ± 1.7 a	14.8 ± 2.9 b	6.8 ± 2.3 de	14.3 ± 1.7 b	9.3 ± 2.1 cd	5.1 ± 1.0 e	9.1 ± 0.6 d
Tyrosine	17.0 ± 1.2 a	10.4 ± 0.6 cd	17.8 ± 1.4 a	12.3 ± 2.8 bc	5.9 ± 1.9 e	13.6 ± 1.7 b	7.9 ± 1.3 de	5.2 ± 0.6 e	10.0 ± 0.6 cd
Valine	20.9 ± 1.7 a	6.7 ± 3.3 c	16.2 ± 1.5 b	13.1 ± 3.8 b	3.8 ± 1.8 cd	12.1 ± 1.7 b	7.6 ± 2.5 c	1.1 ± 0.9 d	5.5 ± 0.7 c
Total	470.0 ± 33.0 a	408.8 ± 19.2 a	447.4 ± 24.7 a	305.4 ± 49.9 b	185.6 ± 50.3 c	316.1 ± 63.0 b	202.8 ± 40.4 c	111.0 ± 27.0 d	161.1 ± 15.9 cd
Bioactive Amines									
Spermidine	7.8 ± 0.6 ab	6.4 ± 0.4 c	8.5 ± 1.1 ab	9.6 ± 1.2 a	7.7 ± 1.2 bc	9.4 ± 1.0 a	7.5 ± 1.0 c	5.0 ± 0.7 d	7.9 ± 0.3 bc
Ammonium ion	4.4 ± 0.4 a	3.0 ± 0.2 b	2.8 ± 0.1 bcd	5.5 ± 0.9 a	2.0 ± 0.7 cde	2.9 ± 0.5 bc	3.4 ± 0.5 b	1.4 ± 0.3 e	2.1± 0.1 de

Mean values were calculated using zero as nd (not detected). Mean values with different letters in the same line are significantly different (Tukey test, p≤0.05)

3.3 (sliced) 11.6 (sliced fresh) to 36.8 (fresh intact) mg/100g (dry matter) of putrescine in mushrooms and only cadaverine 3.6 mg/100g (dry matter) on the mushroom. Thus, the storage temperature can influence the content of bioactive amines in mushrooms.

Eighteen amino acids were analyzed in *Agaricus bisporus* samples and aspartic acid, alanine, arginine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, phenylethylamine, proline, serine, threonine, tyrosine and valine were identified in its free form in the mushroom. Only cystine, methionine and tryptophan were not identified. The acid conditions of the extraction may have led to the degradation of tryptophan and so the non-identification of this amino acid. The levels of tryptophan, as well as of methionine, in *A. bisporus* were identified in low quantities (Tsai et al., 2007; Jaworska & Berna, 2013; Liu et al., 2014; Rotola-Pukkila et al., 2019). As Rotola-Pukkila et al. (2019), cystine was not identified in *A. bisporus* mushrooms; however, this amino acid was identified in *A. bisporus* by other authors (Chiang et al., 2006; Fei et al., 2007; Kim et al., 2009; Jaworska & Berna, 2013; Liu et al., 2014).

Protein amino acids are subunits of protein structure, making a total of 20 different substances, classified according to human nutritional need as essential, non-essential and conditionally essential. The absence or inadequate ingestion of any of the essential amino acids in the diet can lead to a negative nitrogen imbalance, which can lead to weight loss, impair growth, and other clinical symptoms (Marchini et al., 2016). Free amino acid analysis in food matrix underestimates the nutritional value by not determining the amino acid content that is released during the passage of food through the gastrointestinal tract. However free amino acids are substrates for formation of amines with neuroactive and vasoactive activity (Gloria 2005; EFSA, 2011; Fiechter et al., 2013, Papageorgiou et al., 2018) and are non-volatile taste compounds in foods (Mau et al., 2001; Yang et al., 2001).

The presence of bioactive amines in foods is related to intrinsic and extrinsic characteristics. In unfermented foods, the risk levels of biogenic amines such as histamine, tyramine, phenylethylamine, putrescine and cadaverine are related to the action of decarboxylase enzymes from microorganism contamination. In the mushroom *Agaricus bisporus*, although presenting free amino acids histidine, tyrosine, phenylalanine and lysine, it did not present biogenic amines as histamine, tyramine, phenylethylamine and cadaverine, which are formed directly by the decarboxylation of these amino acids (Gloria 2005; EFSA, 2011; Fiechter et al., 2013; Papageorgiou et

al., 2018). Thus, these mushrooms have potential for the formation of biogenic amines, however, the absence of these compounds reflects in the hygienic sanitary quality of the raw material and the processing (EFSA, 2011; Fiechter et al., 2013). Proline and methionine contents found in mushroom samples may be related to the high content of spermidine in mushrooms since these amino acids are involved in the synthesis of polyamines. Proline and methione are envolved in the polyamine biosynthesis. Proline is an intermediary of ornithine when decarboxylated by Ornithine decarboxylase enzyme lead formation of putrescine. Concerning methionine when decarboxylated by the enzyme SAMDC (AdoMetDC) releasing propylamine groups for polyamine synthesis. Arginine may also be related to the formation of bioactive amines through the urea cycle (Gloria, 2005). Regarding the ammonium ions monitored only the preservation process there was a significant decrease in this content.

The amino acid content varied from 408.8 to 470.0 mg/100g in fresh sample, 185.6 to 316.1 mg/100g in cooked sample, and 111.0 to 202.8 mg/100g in canned samples. When lot was evaluated, the fresh samples of lot A, B and C did not differ significant. However, in cooked samples from lot A and C did not differ significantly in the total free amino acids content, but showed significant differences with lot B. A different behavior was observed in the canned samples. When the total amino acids lot A was higher than and B samples and lot C samples had not significant differences with lot A and B. When the processing (cook and preserve) is evaluated, in each separate lot there was a significant decrease in the total free amino acids content.

The amino acids that more contributed to the total free amino acids content were alanine (ranging from 91.6 to 105.7 mg/100g in fresh samples, from 40.3 to 66.6 mg/100g in cooked samples and from 24.5 to 51.0 mg/100g in canned samples); glutamic acid (ranging from 94.2 to 98.6 mg/100g in fresh samples, from 41.7 to 56.9 in cooked samples and from 16.5 to 33.0mg/100g in canned samples); and proline (ranging from 38.4 to 89.8 mg/100g in fresh sample, from 24.7 to 49.0 mg/100g in cooked samples and from 15.6 to 19.4mg/100g in canned samples). These data were pertinent to the literature data, in which *A. bisporus* was characterized by the high content of alanine and glutamic acid (Chiang et al., 2006; Fei et al., 2007; Tsai et al., 2007; Kim et al., 2009; Jarwoska & Bernaś, 2013; Liu et al., 2014; Rotola-Pukkila et al., 2019), being proline a differential in the present object.

Within each treatment, only aspartic acid, alanine and glutamic acid, for fresh samples, glutamic acid, for cooked sample, aspartic acid, proline and glycine for canned samples did not present significant differences. The significant differences in the other amino acids can be justified by the high variation content between the lots. In general, the variation between lots was high, being highest in proline, valine and lysine in fresh; glycine, valine and lysine in cooked, valine, lysine and serine in canned samples. The quality of the substrate may have a direct influence on the nitrogen content of the mushrooms (Silva et al., 2007) and, therefore, the free amino acids content. However, studies where the effect of the substrate on the amino acid content was evaluated are scarce. The study of Mendez et al. (2005) showed that mushroom harvest had a greater influence than the substrate. In addition, the storage time also influences the free amino acid content in the mushrooms, which may increase after harvest (Tseng & Mau, 1999). Also, different strains of the same species may present different free amino acid compositions in the fruiting body as observed by Manzi et al. (1999) in the *Pleurotus ostreatus* strains. Therefore, free amino acid content in mushroom may vary due many factors, which explains the difference found between lots, presented in Table 1.

Regarding the total amino acids, there was an average loss of 39.7% in the cooking process and 64.6% in the canned process. The most affected amino acids were glutamic acid, alanine and proline, which had an average loss of 46.6, 41.0 and 42.5%, respectively, in the cooking process. In the canned process, the amino acids with the highest mean loss were valine 71.2%, glutamic acid (75.7%) and serine (73.6%). The high loss of valine content (essential amino acid) by the canned processing may affect the nutritional value of the canned mushroom, however this can only be confirmed when known the valine content released in the gastro intestinal tract after the ingestion of the mushrooms. The thermal process employed in cooking and canned is applied to improve the conditions of digestibility, palatability, prevent the action of microorganisms and inactivate enzymes in order to increase the shelf life of the food. The the use of high temperature in cooking and canned samples may have favored the loss of free amino acids that in from the mushroom to the cooking soup by leaching. The acid condition of the pickle employed in the canned process may have favored the ionization of the free amino acids providing the output from the mushroom to the pickle. Rotola-Pukkila et al. (2019) and Liu et al. (2014) also observed reduction

in free amino acids when thermal processing was used. Rotola-Pukkila et al. (2019) studied the effect of cooking on the species *A. bisporus*. They quantified the amino acid content in the mushroom and in the cooking soup and, for heated mushroom samples, a decrease of free amino acids was observed. Also, when comparing the sum of amino acids content of the cooking soup with free amino acids content in the cooked mushroom to the amino acid content in fresh mushroom, it is possible observe that the sum of amino acid released in the cooking soup and the amino acid in cooked mushroom is higher than the amino acid content determined in fresh. In this way it is possible to conclude that in addition to the normal loss due to leaching by the cooking soup, the heat treatment causes protein hydrolysis and consequently loss of amino acids. (Li et al., 2011; Liu et al., 2014).

The influence of processing on the preservation of amino acids in mushroom was a target of some studies. Liu et al., 2014 observed that frozen mushroom had more free amino acids than canned and salted mushroom. Unconventional processing was also applied to the preservation of mushrooms, Pei et al. (2014) presented the microwave freeze drying as a new alternative for preservation and maintenance on the amino acid content of mushrooms. Fei et al. (2017) introduce the ultrasonic osmotic dehydration that can retaining more free amino acids in mushroom. Shcheglova & Vereshchagin (2015), investigated the influence of vacuum-pulse drying which leads to increasing free amino acids content and reducing the activity of trypsin inhibitors in edible mushrooms.

According to Mau et al. (2001) and Yang et al. (2001) classification, free amino acids in mushrooms are divided based on taste characteristics in four groups. Group one was monosodium glutamate-like (MSG-like) or palatable taste amino acids, including aspartic and glutamic acids. Group two presented sweet taste amino acids, including alanine, glycine, proline, serine and threonine. The third class belongs to bitter amino acids, including arginine, histidine, isoleucine, leucine, methionine, phenylalanine, and valine. Last of all, lysine and tyrosine contributed to tasteless mushrooms. The amino acids MSG-like and sweet can be responsible for the pleasant taste in the mushrooms. The characteristics of the mushrooms taste with respect to the free amino acids are presented in **Table 2.**

Table 2. Profile and levels (mg/100g) of amino acid taste like in three kinds of process of *Agaricus bisporus* mushrooms*.

Process/Lot	Amino acid Taste like			
	MSG-like	Sweet	Bitter	Tasteless
Fresh				
A	118.7 ± 7.8 a	204.6 ± 15.5 a	110.5 ± 7.9 a	36.1 ± 2.5 a
B	110.6 ± 7.1 a	227.7 ± 11.3 a	57.4 ± 3.0 b	13.2 ± 0.7 d,e
C	115.2 ± 4.5 a	207.7 ± 9.8 a	97.5 ± 8.8 a	27.0 ± 2.2 b
Cooked				
A	70.9 ± 9.7 b	135.5 ± 16.9 b	74.3 ± 18.6 b	24.8 ± 4.7 b
B	50.7 ± 7.1 c	98.8 ± 15.0 c	32.4 ± 8.4 d	9.7 ± 2.0 e
C	64.8 ± 8.6 b	150.3 ± 36.6 b	73.7 ± 9.2 b	20.7 ± 2.4 c
Canned				
A	42.6 ± 4.8 c	96.6 ± 23.5 c	48.8 ± 10.0 c	14.8 ± 2.1 d
B	27.0 ± 3.8 d	54.3 ± 13.5 d	21.7 ± 4.8 e	8.1 ± 1.3 f
C	23.6 ± 7.4 d	81.2 ± 7.7 c	43.0 ± 3.1 c	13.2 ± 1.1 d,e

Mean values with different letters in the same column are significantly different (Tukey test, p≤0.05). MSG-like: monosodium glutamate-like; Aspartic acid + Glutamic acid. Sweet: Alanine + Glycine + Serine + Proline. Bitter: Arginine + Histidine + Isoleucine +Leucine +Phenylalanine + Valine. Tasteless: Lysine+ Tyrosine.

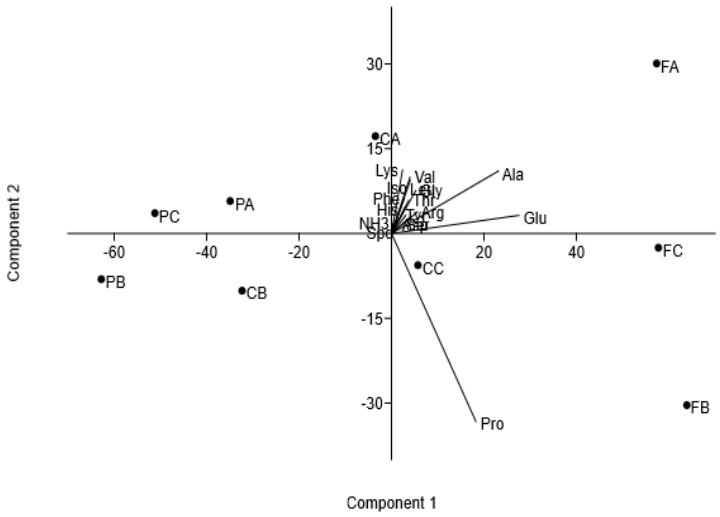
As identified in the work of Tsai et al. (2007), *Agaricus bisporus* mushrooms possessed highly intense umami taste mainly due to the content of glutamic acid. This finding might explain why mushrooms have long been used as a food or food-flavoring material. However, the processing considerably reduces the free amino acid content and, as a consequence, increases the loss in flavor compounds. Also, sensorial studies are needed to define the taste threshold of free amino acids in mushrooms, and consequently the impact of the processing on the taste.

3.1.2. Multivariate analysis

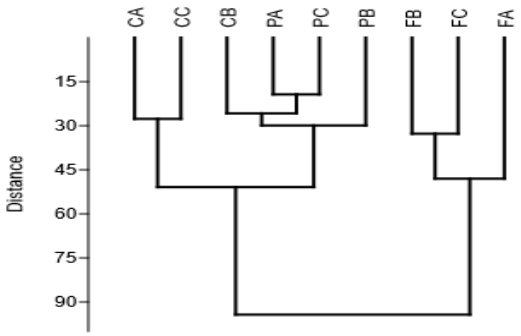
The chemometric analyzes applied to estimate influence of the process (cook and preserve) on the content of bioactive amines, amino acids and ammonium ion in three lots of *A. bisporus* mushrooms are represented in **Figure 2**. Before PCA model was built, data were auto scaled, and no other preprocessing was used. The principal component (PC) model was built with the two principal components (PC1 and PC2), explain 97.2% of the X-variance. The first PC explained 86.8% of all model variance,

and as can be seen in **Figure 2a**, it differentiates Fresh samples (FA, FC and FB) and Cooked sample of lot C (positive values) from the cooked sample of lot A and B (CA and CB) and all canned samples. The fresh samples were more distant from the PC1 axis since these samples suffered no losses in spermidine and amino acids from processing. The cooked samples of lot A and C (CA and CC) presented intermediate position (close to 0 in x-axis), however the cooked sample of lot B was present in low value in the x-axis together with the canned samples. Thus, the canned processing provides big losses in amino acids (Rotolla-Pukkila- 2019), spermidine and ammonium ions content. However, large differences can be seen in different lots of mushrooms and can be accentuated by processing. PC2 explained 10.5% of the model variance, and it differentiates the samples of the lot A (FA, CA and PA) and the canned sample of lot C (PC) (positive values) from all other samples. **Figure 2a**. Through the evaluation of PC2 loadings it can be seen that proline differentiate FB, CC, FC, CB and PB from PC, PA and CA that have higher contents in Glu and Ala.

The samples were grouped by Hierarchical Cluster Analysis using algorithm Paired group (UPGMA) and the similarity Euclidean index that explaining 84.5% of the X-variance. As can be seen on the resulting dendrogram in Figure 2b, the resulting clustering can be explained by the different mushroom lots and process. Fresh samples cluster togethers, the FA sample present greater distance clustering than FB and FC sample. That way lots B and C of fresh samples are more similar. However, after cook process, the sample CA and CC were more similar (clustering together) that CB, which cluster together that the canned samples. Chemometric analyzes was also applied to estimate influence of the process (cook and preserve) on the flavours provide of amino acids in three lots of A. bisporus mushrooms are represented in **Figure 3**.



(a)



(b)

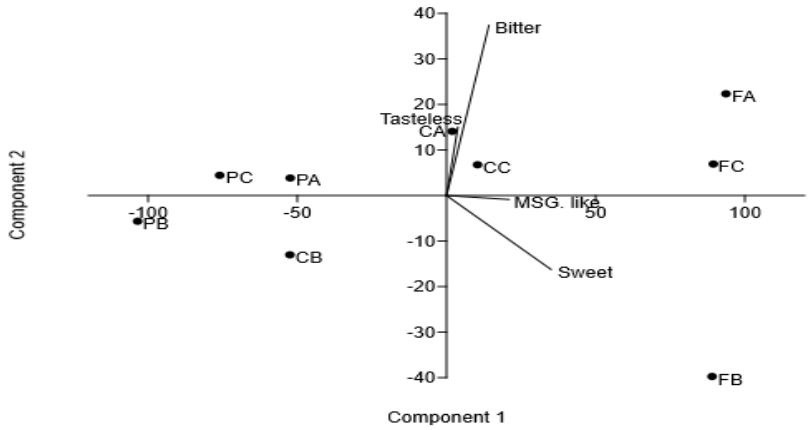
Figure 2. Scatter plot (a) and dendrogram (b), obtained by Principal Component Analyses and Classical Cluster Analyses, for the mean of bioactive amines, free amino acids and ions ammonium for observations obtained at each mushroom process. FA = fresh mushroom lot A, FB = fresh mushroom lot B, FC = fresh mushroom lot C, CA = cooked mushroom lot A, CB = cooked mushroom lot B, CC = cooked mushroom lot C, PA = canned mushroom lot A, PB = canned mushroom lot B, PC = canned mushroom lot C, Ala = alanine, Asp = aspartic acid, Glu = glutamic acid, Gly = glycine, His = histidine, Ile = isoleucine, Leu = leucine, Lys = lysine, Met = methionine, NH4+ = ammonium ion, Phe = phenylalanine, PRO = proline, Ser = serine, Thr = threonine, Tyr = tyrosine, Val = valine and Spd = spermidine.

Before PCA model was built, data were auto scaled, and no other preprocessing was used. The principal component (PC) model was built with the two principal components (PC1 and PC2), explain 94.2% of the X-variance. The first PC explained 94.2% of all model variance, and as can be seen in Figure 3a, it is possible observe the same distribution presented in the previous PCA (Figure 2a), the contents of amino acids in mushrooms was the major factor in the principal component 1 (X-axis). It is possible to observe a reduction in the content of free amino acids in cooked mushrooms.

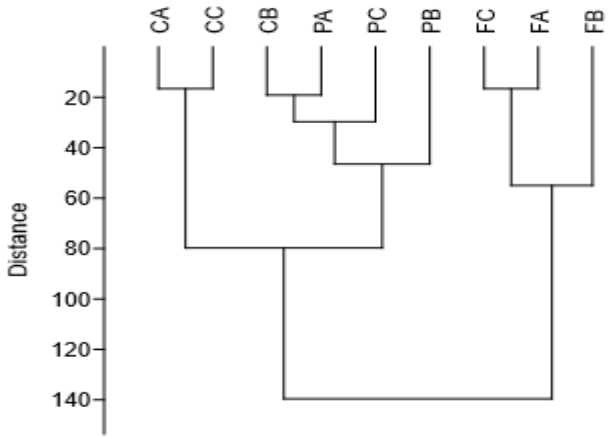
Thus, the high loss of amino acids in canned process (Table 1) will also lead to a greater loss in amino acids responsible for the flavor of that mushrooms. PC2 explained 5.1% of the model variance, and it differentiates Bitter and tasteless amino acids (positive values) than Sweet and MSG-Like (negative values) in mushrooms. It is possible to notice that the samples from lot B have more sweet amino acids than samples from lot A, which is characterized by the Bitter flavor. Sample C occupies intermediate position presenting more proximity to the MSG-like vector.

The samples were grouped by Hierarchical Cluster Analysis using algorithm Paired group (UPGMA) and the similarity Euclidean index that explaining 83.4% of the X-variance. As can be seen on the dendrogram in Figure 3b, the resulting clustering can be explained by the different mushroom lots and process. As in the previous dendrogram (Figure 2b) the samples were grouped into two large groups, the first are related to fresh samples and the other are related to processed samples (cooked and canned). FA and FC samples were grouped together at a shorter distance than FB sample. When cooked samples were evaluated, the lot A sample (CA) was grouped with lot C sample (CC). The same did not happen for lot A canned samples (PA), which was grouped with sample CB. The PC sample presented greater distance, but it was grouped together the CB and PA samples. The sample PB presented the largest distance in the dendrogram since lot B presented the lowest values and was more affected by the processing (cooked and canned).

The Principal Component Analysis allows the graphical visualization of the effect of the processing and the lot on the content of amino acids in the mushrooms. It is possible to conclude that the influence of the processing is greater than the influence of the lot in relation to the total content, since the processing by itself leads to a reduction in the amino acids content in the mushrooms. The influence of the lot is



(a)



(b)

Figure 3. Scatter plot (a) and dendrogram (b), obtained by Principal Component Analyses and Classical Cluster Analyses, for the mean of flavour amino acids group (MSG-like, Sweet, Bitter, Tasteless) in mushroom process. FA = fresh mushroom lot A, FB = fresh mushroom lot B, FC = fresh mushroom lot C, CA = cooked mushroom lot A, CB = cooked mushroom lot B, CC = cooked mushroom lot C, PA = canned mushroom lot A, PB = canned mushroom lot B, PC = canned mushroom lot C, Ala = alanine, Asp = aspartic acid, Glu = glutamic acid, Gly = glycine, His = histidine, Ile = isoleucine, Leu = leucine, Lys = lysine, Met = methionine, NH4+ = ammonium ion, Phe = phenylalanine, Pro = proline, Ser = serine, Thr = threonine, Tyr = tyrosine, Val = valine and Spd = spermidine.

related to the composition in the individual content of each amino acid in the mushrooms. When performing clustering it is possible to conclude that the samples will be grouped principally by processing (cook and canned). With the exception of the CB sample, due to the reduced amino acid content, the samples that went through the canned processing were grouped. It is possible to perceive the same behavior with regard to taste amino acids. In which similar samples (same treatment) are grouped together. However, the taste of the cooked mushroom from lot B may not resemble the taste of a canned mushroom (as shown in the figure 3), but it is possible to conclude that depending on the lot the loss of taste amino acids of the mushroom by the cooking process can resemble the loss of taste compounds by the canned process.

4. Conclusion

Although free amino acids were identified as precursors of bioactive amines in *A. bisporus* mushroom, only spermidine was identified in this mushroom. This confirms the hygienic sanitary quality of *A. bisporus* acquired at Ceasa in the city Campinas-SP, Brazil. Polyamine content of *A. bisporus* may vary depending on the different lots of mushroom. Regarding the processing, spermidine content is maintained in the cooking process, but it decreases in the canned processing. However, this processed mushroom is still considered as a food with high content of polyamines. The absence of other bioactive amines favors this mushroom to be the object of new studies in order to develop foods and formulations with highest spermidine content.

Free amino acid profile in *A. bisporus* did not vary in function of the different mushroom lots and processing. The content of free amino acids in mushrooms may vary depending on the lot and the processing. Cook and preserve process led a loss of free amino acids in mushrooms, and the loss of free amino acids was higher in canned mushroom. The success of *A. bisporus* in cooking is related to the umami and sugar taste amino acid content, despite the reduction in the taste of the mushrooms due to the process. Therefore, canned processing methods should be improved in order to maintain amino acid content and improve the non-volatile flavor.

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CAPÍTULO IV: The effect of *in vitro* digestion on spermidine and amino acids release from fresh and processed *Agaricus bisporus* mushroom

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Abstract

Amino acid and bioactive amines in mushroom may be modify by process and these compounds could be released after digestion. UHPLC method was used for the simultaneous determination of amino acids and biogenic amines in mushrooms. 14 amino acids were detected in the free form in fresh mushroom. Alanine and glutamic acid were the amino acids with the highest contents. The content of spermidine remains after the cooking, decrease after canning process and remained the same after the *in vitro* digestion. Arginine and methionine were detected only after the *in vitro* digestion. The digestion process is able to release much of the protein amino acids. Multivariate analysis showed that the protein hydrolysis in the processed mushrooms is very small in the gastric phase. The bioaccessibility of spermidine in *A. bisporus* able the consumption of this food to contribute to the daily requirement of polyamine.

Key words: Polyamines, bioaccessibility, digestibility, essential amino acids

1. Introduction

The world consumption of edible mushrooms has increased in the last years. According to the Food and Agriculture Organization (FAO) of the United Nations, the mushroom production was 5.9 million tons in 2007 and it increased to 10.2 million tons in 2012, presenting a growth of more than 73% in ten years (FAOSTATS, 2018). In addition to the economic value, mushrooms have a small environmental footprint, as they grow from agricultural and forest wastes and require relatively little water or land. Furthermore, they can be used as agents of environmental management, by using natural resources in a less destructive way for the biosphere and by promoting sustainable development in all ecosystems (Donnini et al., 2013; Feeney, Dwyer, Hasler-Lewis, Milner, Noakes, Rowe, 2014). Several mushroom species are commercially available; however, *Agaricus bisporus* represents 15% of global world's mushroom supply (Royse, Baars, & Tan, 2017).

Mushrooms are known to contain large amounts of dietary fibers, minerals (K), vitamins (provitamin D2, vitamin B12) and essential amino acids (Kalač, 2013; Manninen, Rotola-Pukkila, Aisala, Hopia, & Laaksonen, 2018); moreover, they provide few calories due to low fat contents. Mushrooms are also valued due to functional properties attributed to many compounds, including bioactive amines (Nishimura et al., 2006; Nishibori et al., 2007; Dadáková et al., 2009) and amino acids (Chen et al., 2015; Fei et al., 2017; Poojary et al., 2017; Dong et al., 2018; Rotola-Pukkila et al., 2019). These nitrogenous compounds are also relevant in the sensorial characteristics of mushrooms (Mau, Lin & Chen, 2001; Yang, Lin, & Mau, 2001).

The occurrence and levels of bioactive amines in cultivated mushrooms are described in the literature (Yamamoto, Itano, Kataoka, & Makita, 1982, Dadáková et al., 2009). High spermidine contents were detected in mushroom, followed by agmatine, putrescine, tyramine, tryptamine and phenylethylamine (Dadáková, Nova, & Kalač, 2009). The polyamines (spermidine and spermine) play various physiological functions, including stimulation of cell division and proliferation, gene expression for the survival of cells, DNA and protein synthesis, regulation of apoptosis, oxidative stress and angiogenesis (Lenis, Elmetwally, Maldonado-Estradam, & Bazer, 2017). Also, polyamines are effective in wound healing, modulation of the permeability and renewal of the intestinal mucosa, affecting the uptake of nutrients and allergenic proteins (Kalac & Krausová, 2005; Gloria, 2005; Kalac, 2014).

The contribution of polyamines to cell regeneration may be considered undesirable in some pathological conditions, since they are frequently present in high concentrations in rapidly dividing cells and growth tissues, being associated with tumor growth, tumor invasion and metastasis. It is important to mention that polyamines are not related to the initial development of cancer (Gloria, 2005; Kalac and Krausová, 2005; Kalac, 2014). High levels of free amino acids are also found in commercial mushrooms. Amino acids are important because their role in human health (FAO, 2007; Levesque, Moehn, Pencharz, & Ball, 2010; Marchini et al., 2016) and mushroom sensory properties (Mau et al., 2001; Yang et al., 2001; Poojary et al., 2017; Rotola-Pukkila et al., 2019). Mushrooms are rich in umami amino acids – is highlighted the high free glutamic acid content in *A. bisporus* mushroom (Mau et al., 2001; Yang et al., 2001; Poojary et al., 2017; Rotola-Pukkila et al., 2019). According to Tsai, Tsai, & Mau, 2006) the *A. bisporus* mushrooms highly intense umami taste explain why these mushrooms have long been used as a food or food-flavoring. Aspartic acid also contributes to umami taste and it was quantified free in *Agaricus bisporus* (Rotola-Pullika et al., 2019). Other free amino acids sweet (alanine + glycine + serine), bitter (arginine + histidine + isoleucine +leucine +phenylalanine + valine) taste and tasteless (lysine + tyrosine) were also identified free in *A. bisporus* (Kim et al., 2009; Jawoska and Bernas 2013; Rotola-Pukkila et al., 2019).

Amino acids are the most important building blocks of body tissues, enzymes and hormones, so they are indispensable for vital functions. When it comes to metabolic dysfunction, insufficient resorption, increased nutritional demand after surgical trauma, and medical care, there is benefic evidence for amino acids targeted supplementation (Cruzat, Krause, & Newsholme, 2014; Jones, Rivera, Puccinelli, Wang, Williams, & Barber, 2014; Lukey, Katt, & Cerione, 2017). However, in order to know the effective nutritional value of a food, it is necessary to know, beyond the profile and contents of free amino acids, it is important to know the fractions released from proteins by digestion. The bioaccessibility of bioactive amines and amino acids is a valid tool to access the nutritional value of mushrooms.

Bioaccessibility is a more complex food analysis, which can be defined in the fraction of a constituent that is released from a food matrix in the gastrointestinal tract and become available for absorption (Ariza et al., 2018). The determination of the *in vitro* bioaccessibility of bioactive amines and amino acids in mushroom would be interesting, once emerge a hypothesis that bioactive amines and amino acid could be

in the bound form in mushroom and could be released after *in vitro* digestion. Also, the process may impact the bioaccessibility (Ariza et al., 2018; Cilla, Bosch, Barberá, & Alegría, 2018; Mercadante & Mariutti, 2018).

Thus, the objective of this study was to investigate the *in vitro* bioaccessibility of fresh and processed *Agaricus bisporus* mushroom regarding bioactive amines and amino acids.

2. Material and methods

2.1. Sample and reagents

Agaricus bisporus mushrooms (5 kg) were purchased from distributors at Ceasa, Belo Horizonte, MG, Brazil. Bile salts, pancreatin, pepsin from pig gastric mucosa, thimerosal, orthophthaldialdehyde were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The bioactive amines (spermidine trihydrochloride, spermine tetrahydrochloride, agmatine sulfate, putrescine dihydrochloride, cadaverine dihydrochloride, histamine dihydrochloride, tryptamine, serotonin hydrochloride, tyramine hydrochloride, 2-phenylethylamine hydrochloride), L-amino acids (alanine, arginine hydrochloride, asparagine, aspartic acid, cystine, glycine, glutamic acid, glutamine, histidine hydrochloride, isoleucine, leucine, lysine hydrochloride, methionine, phenylalanine proline, serine, threonine, tryptophan, tyrosine, valine, and norvaline - internal standard), and ammonium chloride standards were also from Sigma Chemical Co. (St. Louis, MO, USA). AccQ.FluorTM pre-column derivatization kit was purchased from Waters (Milford, MA, USA).

The reagents were of analytical grade, except acetonitrile, which was LC grade. Ultrapure water was obtained from a Milli-QTM system (Millipore Corp., Milford, MA, USA). The organic and aqueous solvents for the UPLC analysis were filtered through 0.22 µm pore size HAWP and HVWP membranes, respectively (Millipore Corp., Milford, MA, USA).

2.2. Mushroom processing

Fresh mushrooms were purchased at commercial maturity directly from distributors in the market of Campinas, state of São Paulo, Brazil and were divided into

three sublots - fresh, cooked and canned mushrooms). Fresh mushrooms were analyzed immediately for amino acids and bioactive amines. Cooked mushrooms were prepared by heat treatment of 100 g fresh mushrooms in 1 L boiling water (97 ºC) for 10 min (Soller-Rivas et al., 2009). To prepare canned mushrooms, the mushrooms were washed, allowed to soak in 1% sodium bisulfite for 2 min and cooked in boiling water (97 ˚C) for 5 min. The cooked mushrooms (100 g) were drained and placed into 250 mL glass jars, which were filled with 3% NaCl and 0.12% ascorbic acid solution at 90 ˚C, sealed, pasteurized at 97 ˚C for 20 min and cooled to room temperature (EMBRAPA, 2000).

2.3. Methods of analysis

2.3.1. Moisture and crude protein contents

The moisture and crude protein contents of fresh, cooked and canned mushrooms were obtained by oven-drying at 105 °C and micro-Kjeldahl, respectively (AOAC, 2012). Crude protein was calculated as nitrogen content × 4.38 (Kalač, 2013).

2.3.2. *In vitro* protein digestibility

The determination of *in vitro* protein digestibility of fresh, cooked and canned mushroom was performed by measuring the hydrolysis of each sample, after treatment with an enzyme sequence consisting of pepsin (Sigma P-6887) and pancreatin (Sigma P-3292) (Akeson & Stahman, 1964; Tavano, Neves, & Da Silva Junior, 2016). Mushroom samples were weighted in each tube to obtain around 50.0 mg of protein. The sample tubes were six, three tubes for samples with enzymes and three tubes for samples without enzymes. Three other tubes were prepared without any sample addition, and more three tubes were prepared for the blank of the enzymes (containing only buffers). Seven milliliters of 0.1 mol/L KCl-HCl buffer, pH 1.5 and 0.5 mL of pepsin solution (1.5 mg pepsin/mL in KCl-HCl buffer, 1 mol/L, pH 1.5) or 0.5 mL of buffer (blank) were added to each tube. After incubation in a water bath at 37 °C for 3 h, to each tube, were added 3.0 mL of 0.2 mol/L sodium phosphate buffer, pH 8.0, 3.0 mL of 0.1 mol/L sodium phosphate buffer, 80 µL of thimerosal (Sigma T-5125) and 0.5 mL

of pancreatin solution (4.0 mg/mL 0.1 mol/L phosphate buffer). After 24 hours of incubation, the reaction was stopped by addition of 3.0 mL of 60% trichloroacetic acid - TCA and the tubes were centrifuged (7,000 *g*/ 30 min). The supernatants were filtered in volumetric flasks and the volumes were adjusted to 25.0 mL.

The percent hydrolysis of each sample was quantified by determining the amino acids in the supernatant of the digests after precipitation with 10% trichloroacetic acid (TCA) by reaction with OPA reagent (25.0 mL of 100.0 nmol/L sodium tetraborate, 2.50 mL of 20% sodium dodecyl sulfate, 40.0 mg of ortho- phthalaldehyde in 1.0 mL of methanol, 100 µL of mercaptoethanol, final volume of 50.0 ml adjusted with distilled water). The reaction with OPA reagent was made by adding 1.0 mL of OPA reagent directly to the aliquots of 0-130.0 µL of supernatant sample.

After exactly 2 min of reaction, the absorbances were read at 340 nm against a reaction blank. An analytical curve of the amino acid L-leucine was prepared as reference and the degree of hydrolysis, calculated as the percentage of free amino acids in the supernatant, expressed as L-leucine mols, was compared to the total of amino acid present in 50.0 mg of each sample, estimated on the basis of the average molecular weight of amino acids (MW = 113), according to the following formula:

$$\%H\ opa = \frac{(AAs - AAba - AAbe)}{AAtm}\ x\ 100$$

Where:
AAs = mols of amino acids in the sample supernatant;
AAba = mols of amino acids in the supernatant of the blank of sample;
AAbe = mols of amino acids in the supernatant of the blank of enzymes;
AAtm = mols of amino acids in the sample supernatant considering the average molecular weight of the amino acids (1 mol of amino acids = 113.0 g);

2.3.3. Profile and levels of bioactive amines, amino acid and ammonium ions in mushrooms

Bioactive amines, free amino acids and ammonium ions were extracted from 2 g of fresh, cooked and canned mushrooms with 3 mL of 1.6% TCA. The samples were agitated for 2 min in a shaker at 200 rpm and centrifuged at 7,000 *g* for 4 min at 4 °C. This step was repeated twice. The supernatants were collected and filtered through a Whatman #1 filter in a volumetric flask. Then, 40 µL of L-norvaline 50 mmol/L (internal standard) was added and the volume was adjusted to 10 mL. For the *in vitro* digestion

analyzes, 4 µL L-norvaline 50 mmol/L (internal standard) was added to 996 µL of each *in vitro* digestion fraction. An aliquot of the extract and *in vitro* digestion fractions (500 µL) were neutralized using 300 µL of 0.1 mol/L NaOH. After homogenization, 5 µL of the neutralized extracts were mixed with 30 µL of AccQ.Fluor® borate buffer and 15 µL AQC, it was allowed to rest for 1 min and then, it was heated in a water bath at 55 °C for 10 minutes. The extract was filtered using PTFE 0.22 µm pore size membrane (Minisart SRP 4®, Sartorius, Gottingen, Germany) and analyzed by UPLC.

A Waters AcquityTM Ultra Performance LC (UPLC) system (Waters, Milford, MA, USA) equipped with an AcquityTM tunable ultraviolet (TUV) detector (249 nm) was used, with a CSH C18 column (50 x 2.1 mm, 1.7 µm id., Acquity UPLC). The solvent system consisted of A – 0.01 mol/L sodium acetate adjusted to pH 4.80 with acetic acid and B – acetonitrile at gradient elution. The injection volume was set at 2 µL and the gradient elution was operated at a flow rate of 0.9 mL/min as follows: initial–2.5 min/0–0% B; 2.8–4.5 min/0–3% B; 4.5–10.0 min/3–30% B; 10.0–11.0 min/30–100% B; 11.0–11.75 min/100–100% B; 11.75–12.5 min/100–0% B, and further re-equilibration at initial conditions for another 2.5 min, total cycle time of 15 min until the next injection. The concentration of bioactive amines, amino acids and ammonium ions was calculated by interpolation in the respective analytical curves ($R^2 \geq 0.96$) and the recovery of the internal standard was also used in the calculation.

2.3.4. *In vitro* digestion and potential bioaccessibility/absortion

Fresh, cooked and canned mushrooms were digested *in vitro* according to the method of Ariza et al. (2018) with modifications. Briefly, 5 g of mushroom were mixed with 10 mL of 6 mol/L HCl, pH 1.8. To obtain the gastric fraction, each sample was ground in an Ultraturrax-T-25 (IKA, Staufen, Germany) for 30 s, 3.67 mg of pepsin from pig gastric mucosa was added and it was incubated at 37˚ for 120 min under continuous shaking (Cientec, Belo Horizonte, MG, Brazil). The samples for the gastric digestion study were taken, neutralized with $NaHCO_3$, purified by centrifugation at 2,000 *g* for 10 min (Excelsa Baby II 206-R, Fanen, São Paulo, Brazil) and stored at -80 ˚C. To obtain the intestinal digestion fraction, after the gastric digestion, the pH was raised to 7.8 with $NaHCO_3$ and 45 mg of pancreatin and 281.2 mg de bile salts were added and incubated at 37 ˚C for 120 min under continuous shaking. A blank was also obtained, without addition of sample to eliminate any interferences from the process.

The fractions of sample and blank were purified by centrifugation at 2,000 *g* for 10 min and the supernatant was collected and stored at -80 °C, until analysis of amines, ammonium ion and amino acids. The results were calculated as the indicated below:

(1) $Gastric\ \boldsymbol{in\ vitro}\ digestion =$

$$analytes\ released\ after\ gastric\ \boldsymbol{in\ vitro}\ digestion\ (mg/100\ g)$$

(2) $Gastric\ and\ intestinal\ \boldsymbol{in\ vitro}\ digestion\ (mg/100g)\ =$

$$analytes\ released\ after\ gastric\ and\ intestinal\ \boldsymbol{in\ vitro}\ digestion$$

2.4. Statistical analysis

The Past 3.19 software (UIO, Oslo, Norway) was used to evaluate the normality and significance tests in samples (Shapiro-Wilk normality test, Levene homoscedasticity test, one-way ANOVA followed by Tukey's test at 5% probability). Also, this software was used to evaluate the Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) of the contents of biogenic amines, amino acids and ammonium ions obtained from fresh, cooked and canned mushrooms before and after *in vitro* digestion.

3. Results and Discussion

3.1. Fresh mushroom characterization

The parameters amino acid, bioactive amines, ammonium ion, protein and moisture contents and the degree of protein hydrolysis of fresh, cook and canned *Agaricus bisporus* mushrooms are shown in Table 1. As reported in the literature (Kalač, 2014), fresh *A. bisporus* mushrooms had high moisture content (92.8 g/100 g) and low amounts of protein (1.95 g/100 g). The degree of peptide bounds hydrolysis in fresh mushroom by *in vitro* protein digestibility method was 26.4% (Akeson & Stahman, 1964; Tavano, 2016).

Regarding the bioactive amines, among the ten amines analyzed (spermine, spermidine, agmatine, putrescine, cadaverine, histamine, tryptamine, serotonin, tyramine and phenylethylamine), only spermidine was found in *A. bisporus*

mushrooms. The occurrence of spermidine in *A. bisporus*, as well as in other mushrooms and food matrix, has been reported in the literature (Okamoto, Sugi, Koizumi, Yanagida, & Udaka,1997; Dadaková et al., 2009; Kalač, 2013) and its presence is associated with its diverse and relevant roles on cellular metabolism and growth. Based on the classification proposed by Kalač (2014), the *A. bisporus* mushrooms analyzed in this study can be considered as high (>1 mg/100 g) sources of polyamines.

Concerning the amino acid profile, among 18 amino acids analyzed (alanine, arginine, aspartic acid, cystine, glycine, glutamic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine), 14 (alanine, aspartic acid, glycine, glutamic acid, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tyrosine, valine) was detected in the free form in the analyzed samples.

The presence of 8 free essential amino acids in this mushroom species (histidine, isoleucine, leucine, lysine, phenylamine, threonine, tyrosine and valine) can be related to the nutritional potential of this food as a source of essential amino acids. The absence or inadequate ingestion of any of the essential amino acids in the diet can lead to a negative nitrogen imbalance, which can lead to weight loss, impair growth, and other clinical symptoms (Marchini et al., 2016).

This result is similar to those reported by Tsai et al. (2006), Li, Zhang, Claver, Zhu, Peng, & Zhou (2014), Pei et al., (2014) and Rotola-Pukkila (2019) for the great majority of free amino acids on *A. bisporus* mushroom profile. Arginine, methionine and tryptophan were also detected in free form in *A. bisporus* mushroom by these authors.

The average total free amino acids content in *A. bisporus* mushroom was equivalent to 398.8 mg/100 g. This result is similar to those reported by Tsai et al. (2006) Li et al. (2011), Pei et al. (2014) but lower than total free amino acids levels found by Rotola-Pukkila et al. (2019) for *A. bisporus* mushroom. Alanine (93.8 mg/100 g) and glutamic acid (86.0 mg/100 g) were the amino acids with the highest contents, representing 23.5% and 21.6% of total levels. Alanine is classified like sweet and glutamic acid is classified by its umami taste amino acid. These results are similar to those reported by Li et al. (2011) Rotola-Pukkila et al. (2019); however, in Tsai et al.

(2006) and Pei et al. (2014) studies, histidine was the major amino acid found in *A. bisporus* mushroom.

Regarding free essential amino acids level in *A. bisporus* mushroom, the level ranged from 7.1 mg/100 g (tyrosine) to 29.4 mg/100 g (leucine), which represents 1.8 to 7.4% of the total free amino acids content. The other quantified amino acids had an average content lower than 25.7 mg/100g. Although the mushroom samples presented free amino acids histidine, tyrosine, phenylalanine and lysine, it did not present biogenic amines as histamine, tyramine, phenylethylamine and cadaverine, which are formed directly by the decarboxylation of these amino acids (Gloria 2005; EFSA, 2011; Papageorgiou, Lambropoulou, Morrison, Kłodzińska, Namieśnik, & Płotka-Wasylka, 2018). Thus, these mushrooms have potential for the formation of biogenic amines, however, the absence of these compounds reflects in the hygienic sanitary quality of the raw material and the processing (EFSA, 2011).

With respect to ammonium ions, small amounts of this substance were quantified in fresh mushrooms (2.7 mg/100 g).

3.2. Influence of cooking and canning process

As presenting in Table 1, cooking and canning processes did not affect significantly the mushroom moisture (~93%) and protein content (~1.9%). Also, i*n vitro* protein digestibility method was capable of hydrolyzing approximately 24% of the peptide bounds in processed mushrooms, and processing did not affect hydrolysis. The profile of bioactive amines was not altered by the processing, only spermidine was detected after cooking and after canned process. The average content of this polyamine was maintained after cooking (7.1 mg/100 g) and was reduced to 6.2mg/100 g after the canned process, which represents a 13.9% reduction in spermidine by canning. The polyamines have in their structure free nitrogen groups. In the acid medium of the pickle (pH <4.5) this substance is expected to have increased solubility, which could had caused migration of the polyamine to the aqueous phase.

Table 1. Profile and levels of free bioactive amines, amino acids and ammonium ions (mg/100 g, wet base), moisture and crude protein contents, degree of protein hydrolysis and contribution (%) of amino acid released after in vitro digestion to the protein content in fresh cooked and canned *Agaricus bisporus* samples.

Parameter	*Agaricus bisporus*		
	Fresh	**Cooked (97°C/10 min)**	**Canned (3% NaCl and 0.12% ascorbic acid 97 ˚C for 20 min)**
Amino acid (mg/100g)			
Alanine	93.8 ± 2.1 a	59.8 ± 3.5 b	43.5 ± 0.6 c
Aspartic acid	25.7 ± 0.8 a	14.9 ± 1.0 b	11.6 ± 0.2 c
Glutamic acid	86.0 ± 2.9 a	51.5 ± 3.2 b	54.8 ± 1.1 b
Glycine	10.8 ± 0.4 a	7.4 ± 0.4 b	6.3 ± 0.5 c
Histidine	9.4 ± 0.2 a	6.8 ± 0.6 b	6.0 ± 0.2 b
Isoleucine	17.3 ± 0.7 a	11.2 ± 0.6 b	9.1 ± 0.1 c
Leucine	29.4 ± 0.9 a	18.9 ± 1.1 b	15.7 ± 0.1 c
Lysine	19.0 ± 0.6 a	15.1 ± 0.6 b	12.5 ± 0.1 c
Phenylalanine	20.2 ± 1.0 a	12.9 ± 0.7 b	10.5 ± 0.1 b
Proline	19.8 ± 1.0 a	13.2 ± 0.8 b	13.5 ± 0.1 b
Serine	16.0 ± 0.9 a	15.6 ± 1.4 a	14.8 ± 2.7 a
Threonine	24.1 ± 0.8 a	17.2 ± 1.0 b	14.1 ± 0.2 c
Tyrosine	7.1 ± 0.3 c	11.2 ± 0.7 a	9.8 ± 0.2 b
Valine	20.2 ± 0.8 a	13.2 ± 1.1 b	9.5 ± 0.4 c
Total	398.8 ± 13.2 a	268.8 ± 16.4 b	231.9 ± 7.7 c
Bioactive amines (mg/100g)			
Spermidine	7.2 ± 0.2 a	7.1 ± 0.6 a	6.2 ± 0.1 b
Ammonium ion (mg/100g)			
	2.7 ± 0.3 a	2.9 ± 0.3 a	2.7 ± 0.1 a
Moisture content (g/100 g)			
	92.89 ± 0.08 a	92.85 ± 0.12 a	92.81 ± 0.06 a
Crude protein (g/100 g wb)			
	1.84 ± 0.02 a	1.95 ± 0.04 a	1.88 ± 0.01 a
Degree of hydrolysis (%)			
	26.4 ± 3.9 a	24.8 ± 1.8 a	24.3 ± 3.2 a
Amino acid released (%) after in vitro digestion			
	61.1 + 5.6 a	46.5 +1.7 b	45.4 + 2.4 b

Mean values (± standard deviation) with different letters in the same column are significantly different (Tukey test, p≤0.05). 4.38 was used to calculate protein from total nitrogen. 2 wb = wet basis

Despite this loss, canned mushrooms still have high content of polyamines. This is important once dietary polyamines are essential for the maintenance of normal growth, maturation of the intestinal tract (Ali, Poortvliet, Strömberg & Yngve, 2011) and advantageous during the periods of wound healing, post-operational recovery, liver regeneration, or compensatory growth of the lung or the gut (Kalač, 2014). Regarding amino acids, total free amino acids content significantly reduced after cooking (268.8 mg/100 g) and canning process (231.9 mg/100 g) which represents a reduction of 32.6% and 41.9% by cooking and canning processes, respectively. These results are

similar to those reported by Li et al. (2011), and Rotola-Pukkila et al. (2019), that observed significant decrease in amino acid content in *A. bisporus* after processing.

Alanine and glutamic acid were the amino acids with the highest contents. After the cooking, 59.8 mg/100 g for alanine and 51.5 mg/100 g for acid glutamic remained in the mushroom, and it represents a reduction of 37.3% and 40.1% in the content of alanine and glutamic acid, respectively. When comparing canned with cooked mushroom, there were no significant differences in glutamic acid content. However, the release of alanine was higher in canned than in cooked, representing a reduction of 53.6% of this amino acid found free in mushroom.

The content of free essential amino acids was also affected by the processing. Histidine and phenylalanine contents were reduced by processing, when compared to fresh mushrooms (Table 1), and there was no difference between these amino acids content in cooking and in canning processes. The processing represented a loss 31.9% and 48.0% of histidine and phenylethylamine, respectively.

Isoleucine, leucine, lysine, threonine and valine contents were also reduced by processment; however, the reduction was higher by canning processing. The loss of these amino acids ranged from 20.5% to 35.3% by cooking and 34.2% to 53.0% by canned process. Unlike the other free essential amino acids, the processing increased the free tyrosine content in the mushrooms compared to fresh mushrooms. The cooking was responsible for an increase of 57.7% and the canned processing was responsible for the increase of 38%.

The thermal process employed in cooking and canning is applied to improve the conditions of digestibility, palatability, prevent the action of microorganisms and inactivate enzymes in order to increase food's shelf life. The use of high temperature in cooked and canned samples may have favored the loss of free amino acids from the mushroom to the cooking soup by leaching. The acid condition of the pickle employed in the canned process may have favored the ionization of the free amino acids providing the output from the mushroom to the pickle. Rotola-Pukkila et al. (2019) and Li et al. (2011) also observed a reduction in free amino acids when thermal processing was used. Thus, because mushrooms are a complex matrix, the losses of free amino acids during the heat process can occur through Maillard reaction between amino acids and reducing sugars together with Strecker degradation of amino acids

resulting in losses of compounds during heating of *A. bisporus* mushroom (Li et al., 2011). In addition to the normal loss due to leaching by the cooking soup, the heat treatment causes protein hydrolysis and consequently loss of amino acids (Rotola-Pukkila, Yang, & Hopia, 2019). The protein hydrolysis can also explain the free tyrosine content increase by the process on *A. bisporus* mushroom.

With respect to the ammonium ions, the average content of this free substance was not affected significantly by the process.

3.3. *In vitro* digestion of fresh mushroom

The release of bioactive amines and amino acids of *A. bisporus* in *in vitro* digestion was also investigated. The results are expressed after the gastric phase digestion and after the end of *in vitro* digestion (gastric and intestinal phase) (Table 2). Of ten bioactive amines analyzed (spermine, spermidine, putrescine, cadaverine, tyramine, tryptamine, agmatine, serotonin, histamine and phenylethylamine), only spermidine was found in A. bisporus mushrooms. The hypothesis that other bioactive amines could be in the bound form in mushroom and could be released after in vitro digestion was not confirmed. Also, the content of this polyamine remained unchanged at the end of the in vitro digestion process, so that polyamine was fully bioaccessible.

The presence of dietary polyamines can have various health benefits, including promotion of intestinal health, since polyamines are responsible for growth, maturation and regeneration of the intestinal mucosa (Kalač, 2014; Ramani, De Bandt, & Cynober, 2014). Then, its fully bioaccessibility after the in vitro digestion of fresh mushroom could contribute after A. bisporus intake to the development of the immune system, wound healing, anti-inflammatory, antioxidant activity and cardioprotective effects (Kalač, 2014; Ramani et al., 2014; De Cabo & Navas, 2016; Handa, Fatima, & Mattoo, 2018; Sharma, Kumar, & Deshmukh, 2018). Del Rio et al. (2018) reported that spermidine was cytotoxic in intestinal cells cultures (lower dose for adverse effect level of 1452.50 mg/kg); however, the dietary free polyamine after the in vitro digestion was far below the toxic concentration.

Table 2. Profile and levels of free bioactive amines, amino acids and ammonium ions (mg/100 g, wet base) in fresh, cooked and canned mushrooms after *in vitro* gastric and gastric and intestinal digestion.

	After gastric digestion			After gastric and intestinal digestion		
Class/Analyte	Fresh	Cooked	Canned	Fresh	Cooked	Canned
Amino acids						
Alanine	148.8 ± 0.6 b	55.6 ± 0.3 d	59.7 ± 0.3 d	168.1 ± 14.7 a	83.4 ± 1.9 c	80.8 ± 6.9 c
Arginine	23.9 ± 1.2 c	nd d	nd d	66.2 ± 9.7 b	81.6 ± 1.4 a	87.8 ± 6.0 a
Aspartic acid	44.8 ± 0.6 b	17.4 ± 0.1 d	17.2 ± 0.3 d	62.9 ± 5.9 a	35.7 ± 1.2 c	32.4 ± 2.6 c
Glutamic acid	138.9 ± 16.0 b	94.1 ± 2.5 c	75.4 ± 1.4 d	200.7 ± 15.5 a	150.8 ± 19.2 b	105.6 ± 9.7 c
Glycine	16.4 ± 0.1 b	7.2 ± 0.4 c	7.8 ± 0.1 c	25.6 ± 2.3 a	16.8 ± 0.3 b	16.7 ± 1.6 b
Histidine	15.3 ± 0.5 c	7.2 ± 0.1 d	6.9 ± 0.1 d	26.3 ± 2.4 a	22.0 ± 0.6 b	21.4 ± 1.5 b
Isoleucine	43.6 ± 1.2 b	12.2 ± 0.1 d	11.9 ± 0.1 d	60.4 ± 5.2 a	37.1 ± 0.7 c	36.3 ± 2.0 c
Leucine	71.6 ± 1.4 c	20.9 ± 0.1 d	19.9 ± 0.1 d	105.5 ± 9.4 a	90.0 ± 1.3 b	93.0 ± 5.1 b
Lysine	39.3 ± 0.1 b	16.7 ± 0.1 c	13.9 ± 0.1 c	80.7 ± 9.8 a	86.4 ± 1.5 a	85.8 ± 3.9 a
Methionine	15.2 ± 0.1 b	nd c	nd c	22.6 ± 2.1 a	14.8 ± 0.3 b	20.9 ± 0.3 a
Phenylalanine	47.1 ± 1.9 b	15.2 ± 0.1 c	14.8 ± 0.4 c	69.9 ± 6.9 a	67.3 ± 2.0 a	71.1 ± 4.8 a
Proline	37.5 ± 0.9 b	13.4 ± 0.1 d	14.6 ± 0.1 d	42.4 ± 3.3 a	20.0 ± 1.0 c	19.4 ± 1.4 c
Serine	24.4 ± 0.3 c	27.1 ± 0.8 c	21.6 ± 0.6 c	41.8 ± 4.3 b	49.4 ± 1.4 a	38.1 ± 3.7 b
Threonine	41.0 ± 2.4 b	18.5 ± 0.1 d	16.6 ± 0.1 d	56.6 ± 6.8 a	36.8 ± 0.8 b	32.2 ± 2.3 c
Tyrosine	4.4 ± 0.7 d	12.5 ± 0.1 c	10.9 ± 0.1 c	21.1 ± 3.6 b	70.4 ± 1.5 a	73.1 ± 4.5 a
Valine	52.2 ± 4.4 b	13.5 ± 0.3 d	11.7 ±0.1 d	73.6 ± 6.1 a	44.0 ± 1.0 c	39.4 ± 2.5 c
Total	740.5 ± 59.0 c	331.5 ± 2.8 d	302.9 ± 2.1 d	1124.5 ± 102.6 a	906.4 ± 33.2 b	853.9 ± 66.8 b
Bioactive amines						
Spermidine	7.7 ± 1.4 a	7.6 ± 0.1 a	6.5 ± 0.1 b	7.4 ± 0.8 a	7.6 ± 0.4 a	6.2 ± 0.1 b
Ammonium ion						
	5.2 ± 0.8 b	3.2 ± 0.1 d	4.0 ± 0.3 cd	6.6 ± 0.8 a	4.5 ± 0.1 bc	4.5 ± 0.3 bc

Mean values were calculated using zero as nd (not detected). Mean values with different letters in the same line are significantly different (Tukey test, p≤0.05).

Concerning the amino acid profile, besides the 14 amino acids detected in fresh mushroom. The total amino acids released after digestion in the gastric phase were equivalent to 740.5 mg/100 g, representing approximately 40% of protein weight of this food. After *in vitro* digestion (gastric and intestinal phase), the total amino acid content released by this process increased to 1124.5 mg/100 g, which represents approximately 61.1% of the protein weight. Thus, although mushrooms have low protein content, the digestion process is able to release much of the protein amino acids.

After digestion in the gastric phase, alanine (148.8 mg/100 g) and glutamic acid (138.9 mg/100 g) were the mayor amino acids, accounting for 20.1% and 18.8%, respectively, of the total content. This behavior prevailed after the end of the *in vitro* digestion process. However, glutamic acid (200.7 mg/100 g) was the most released amino acid after *in vitro* digestion followed by alanine (168.1 mg/100 g), which represented 17.8% and 14.9% of the total amount of amino acids released after mushroom *in vitro* digestion.

Regarding the essential amino acids released from the *A. bisporus* mushroom after digestion in the gastric phase, the essential amino acid content ranged from 4.4 (tyrosine) to 71.6 mg/100 g (leucine), which represented 0.6 to 9.5% for each essential amino acid of the total amino acid content released at this stage of digestion. When compared to the free amino acid content in the mushrooms, digestion in the gastric phase provided an increase in release of most of the essential amino acids, except for tyrosine that had a decrease in its content. This decrease in tyrosine's content may be related to the sensitivity of this amino acid to acidic digestion conditions in the gastric phase. At the end of the digestion, the essential amino acid content ranged from 21.1 (tyrosine) to 105.5 mg/100 g (leucine), which represented a variation of 1.9 to 9.4% for the essential amino acids found.

The release of the histidine, tyrosine, phenylalanine and lysine in mushroom by the *in vitro* digestion process may contribute to the formation of the bioactive amines like histamine, tyramine, phenylethylamine and cadaverine by the decarboxylation of these amino acids through the gut microbiota (Diether & Willing, 2019). Also, proline (42.4 mg/100 g) and methionine (22.6 mg/100 g) contents release after *in vitro* digestion in mushroom samples may be related to the high content of spermidine in mushrooms once these amino acids are involved in the biosynthesis of polyamines.

The first is an intermediary of ornithine when decarboxylated by ornithine decarboxylase enzyme lead formation of putrescine and methionine, when decarboxylated by the enzyme SAMDC (AdoMetDC), release propylamine groups for polyamine synthesis. Arginine may also be related to the formation of bioactive amines through the urea cycle (Gloria, 2005).

Regarding the ammonium ions, there was an increase throughout the digestion process. This increase may be reflecting the influence of digestive enzymes on protein hydrolysis and consequently increased of ammonia release.

3.4. Influence of processing of mushroom on *in vitro* digestion

The profile of bioactive amines, as well as in the fresh mushroom, was not altered after the digestion of the samples that were cooked and canned. Only spermidine was detected. This polyamine was released by digestion in the gastric phase and its content did not increase after digestion in the intestinal phase. In the end of the *in vitro* digestion were released 7.6 and 6.2 mg/100 g in cooked and canned mushrooms, respectively. When compared to the content in fresh mushrooms at the end of the *in vitro* digestion, the content of this polyamine remained unchanged, so spermidine was also fully bioaccessible in processed mushrooms.

Concerning the amino acids studied, the profile remained unchanged after digestion in the gastric phase. However, after digestion in the intestinal phase, arginine and methionine were detected. The hypothesis that emerges is that these two amino acids were released after the pancreatin in the intestinal phase.

In relation to the total amino acid content, 331.9 and 302.9 mg/100 g were released by the cooked and canned mushroom in the gastric digestion, respectively. These represent 17.0% and 16.1% of the protein weight of the cooked and canned mushroom, respectively. Although processing can increase the digestibility and palatability conditions, the loss of free amino acids in the mushrooms by processing was significant for the total content after digestion.

After digestion in the gastric phase of processed mushrooms, the glutamic acid (94.1 - cooked and 75.4 mg/100g - canned) and alanine (55.6 mg/100 g - cooked and

59.7 mg/ 100 g - canned) were the mayor amino acids for the total amino acid content. Glutamic acid contributed to 28.4% and 24.9% of the total amount of amino acids released in the cooked and canned mushroom, respectively. Alanine contributed to 16.8 and 19.7% of the total content of amino acids released in the cooked and canned mushroom, respectively.

After the *in vitro* digestion of processed mushrooms, glutamic acid was maintained with the highest content among the amino acids detected (150.8 - cooked and 105.6 mg/100 g - canned), which represented a contribution of 16.6% and 12.4% to the total content of the mushrooms cooked and canned after digestion. Besides glutamic acid, are also highlighted arginine, leucine, lysine and alanine contents released after digestion, ranging from 81.6 (arginine) to 90 mg/100 g (leucine) released in the cooked mushroom and 80.8 (alanine) at 105.6 mg/100 g (leucine) released in the canned mushroom. These values represent a contribution in the ranges from 9.0 to 9.9% and 9.5 to 12.4% of the total content released after the digestion of the cooked and canned mushroom, respectively.

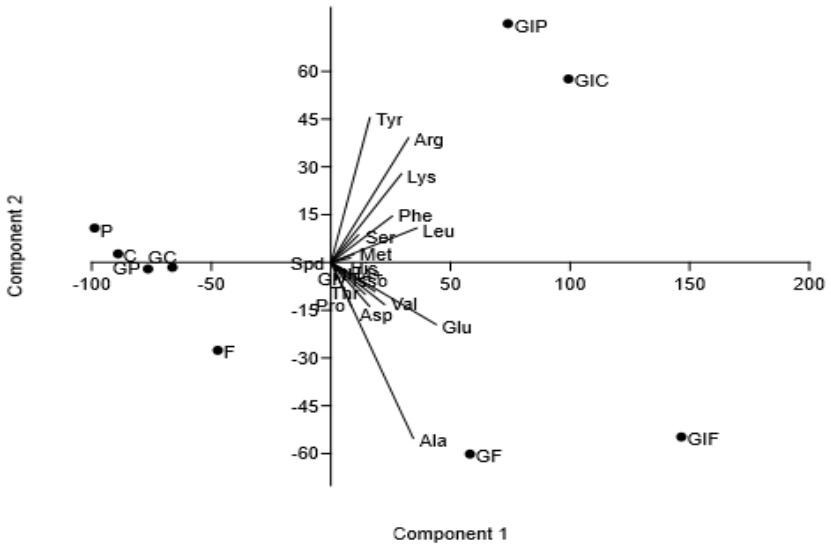
Regarding the essential amino acid contents release after *in vitro* digestion, leucine and lysine were the amino acids that contributed the most to the free amino acid content. Tyrosine and phenylalanine were also detected, representing a contribution percentage of 7.4% (phenylalanine) and 7.8% (tyrosine) in cooked and 7.3% (phenylalanine) and 8.6% (tyrosine) in canned mushroom of the total of amino acids released after *in vitro* digestion. The other essential and non-essential amino acids quantified, presented a contribution for each less than 5% of the total of amino acids released after *in vitro* digestion.

With respect to the ammonium ions, there was an increase during the digestion process, and this may be reflecting the influence of digestive enzymes on protein hydrolysis and consequently increased of ammonia release.

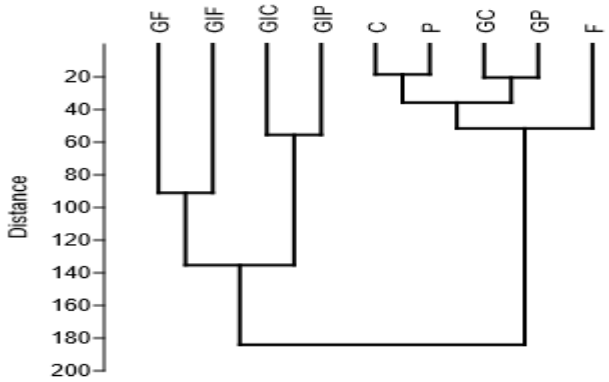
3.5. Multivariate analyses

The multivariate analyzes were applied to estimate *in vitro* digestion influence on the content of bioactive amines, amino acids and ammonium ion in fresh, cooked

and canned *A. bisporus* mushroom. The results are represented in **Figure 1**. Before PCA model was built, data were auto scaled, and no other preprocessing was used. The principal component (PC) model was built with the two principal components (PC1 and PC2), explaining 97.4% of the X-variance. The first PC explained 79.9% of all model variance, and, as can be seen in **Figure 1 (a)**, it differentiates GIP, GIC and GF (positive values of PC1) from the other treatments. When the loadings of PC1 are analyzed, it is possible to see that all variables were positive, which means that all of them contribute to this differentiation. The samples F, P, and C were more distant from the PC1 axis since in these samples only the free amino acids were characterized, and there was no influence of the digestive enzymes present in the *in vitro* digestion method. However, CP and CG were close to these samples, that is, the values of released amino acids by digestion in the gastric phase of cooked and canned samples were very close to the free amino acid values found in the processed mushrooms. In this case, the fresh sample was an exception, since the digestion in the gastric phase was quite influential, presenting significant differences (Table 1) and thus presenting a positive principal component 1. At the end of the digestion, amino acid content significantly increased in all samples, thus GIF, GIC, GIP, had the highest positive values in relation to the principal component 1. PC2 explained 18.4% of the model variance, and it differentiates GIP, GIC, P and C, (positive values) from all other samples Figure 1 (a). Through the evaluation of PC2 loadings it can be seen that Tyr, Arg, Lys, Phe and Leu differentiate GIP, GIP, GIC, PC and C from F, GF and GIF that have higher contents in Glu and Ala. The sample was grouped by Hierarchical Cluster Analysis using algorithm Paired group (UPGMA) and the similarity Euclidean index that explaining 98.2% of the X-variance. As can be seen on the dendrogram in Figure 1 (b), the resulting clustering can be explained by the different mushroom process and in vitro digestion phases. GF and GIF cluster togethers, these samples presented greater distance clustering. GF and GIF are fresh samples and had greater influence of protein hydrolysis by digestive enzymes of gastric phase and gastric phase added to intestinal phase. These treatments were grouped close to GIX and GIP, which were also under



(a)



(b)

Figure 1. Scatter plot (a) and dendrogram (b), obtained by Principal Component Analyses and Classical Cluster Analyses, for the mean of bioactive amines, free amino acids and ammonium ions for observations obtained at each mushroom process and *in vitro* digestion phases. F= fresh mushroom, C = cooked mushroom, P = canned mushroom, GF = fresh mushroom in gastric phase, GC= cooked mushroom in gastric phase, GP = canned mushroom in gastric phase, GIF = fresh mushroom in gastric and intestinal phase, GIC cooked mushroom in gastric and intestinal phase, GIP = canned mushroom in gastric and intestinal phase, Ala= alanine, Asp =aspartic acid, Glu = glutamic acid, Gly = glycine, His = histidine, Ile = isoleucine, Leu = leucine, Lys = lysine, Met = methionine, NH4+ = ammonium ion, Phe = phenylalanine, PRO = proline, Ser = serine, Thr = threonine, Tyr = tyrosine, Val = valine and Spd = spermidine.

the influence of protein hydrolysis by digestive enzymes of gastric phase and gastric phase added to intestinal phase; however, GIX and GIP had natural lowest content of amino acids because they were processed. The samples C, P, GC and GP (C grouped close with P and CG grouped close to GP) were grouped together because they were lower contents in amino acids when compared to F. It is possible to observe in these samples that *in vitro* gastric digestion showed a release of amino acids similar to the content of free amino acids extracted during food sample preparation for analysis before digestion. It may be assumed that the protein hydrolysis in the processed mushrooms is very small in the gastric phase, probably because of amino acids lost and protein degradation in the process. F sample was grouped with samples C, P, CG and GP; however, they are further away. This can be justified because the amino acid content of that sample was not significantly affected since this sample was not processed.

3.6. Nutritional and biological value of *Agaricus bisporus* mushroom

Considering a consumption of 50 g per day of fresh, cooked or canned *A. bisporus* mushrooms, a meal with this food can contribute from 10% to 12.5 % in men and 12.5% to 15 % in women daily requirement intake of spermidine (daily spermidine intake = 30.4 mg/day for men and 25.6 mg/day for women according to Ali et al. (2011). Despite the low protein content found in mushroom, at the end of *in vitro* digestion of fresh and processed mushrooms, of 9 essential amino acids studied, only tryptophan was not identified in the fractions related to *in vitro* digestion of the samples studied, indicating that champignon mushroom (*Agaricus bisporus*) analyzed has the most of the essential amino acids and tryptophan can be ingested in sources like chicken, egg, dairy, meat, sesame seed and sunflower and others (Marchini et al., 2016).

The contribution of essential amino acids in relation to maintenance amino acid pattern of a man weighing 69.4 kg (POF, 2010) found in a 50 g portion of fresh and/or processed mushrooms are too far for an adult human daily necessity, even disregarding peptides that can also be absorbed. However, when the amino acid level released (mg) after *in vitr*o digestion per protein gram of mushrooms is compared to amino acid requirements of adults in mg per g protein (Table 3) (FAO, 2013), these values are adequate for most of the amino acids required, which can represent the great value of the protein found in these mushrooms.

Table 3. Level of released essential amino acids found in a 50 g portion of *Agaricus bisporus* mushrooms (fresh. cooked and canned) after *in vitro* gastric and intestinal digestion. In relation to maintenance amino acid pattern (mg/g protein) for adults (FAO, 2013).

Essential Amino acids	Amino acid level released (mg) after *in vitro* digestion per g protein found in 50 g of mushrooms (wet basis)			Amino Acid Requirements of adults suggested pattern (mg per g protein) *
	Fresh	**Cooked**	**Canned**	
Histidine	14	11	11	15
Isoleucine	32	19	18	30
Leucine	49	45	47	59
Lysine	35	44	43	45
Methionine + Cysteine (SAA)	11	8	11	22
Phenylalanine + Tyrosine (AAA)	44	70	72	38
Threonine	30	19	16	23
Tryptophan	nd	nd	nd	6
Valine	38	22	20	39

SAA, sulphur amino acids; AAA, aromatic amino acids; nd = not detectable.
*Assuming a safe level of protein intake of 0.66 g per kg per day (averaged value for men and women).

4. Conclusions

The hypothesis that other bioactive amines could be in the bound form in mushroom and could be released after *in vitro* digestion was not confirmed. Spermidine has been fully bioaccessible in fresh, cooked and canned *A. bisporus* mushrooms. A 100 g of fresh or processed mushrooms may represent a significant portion of the content of spermidine ingested in the diet. The study of *in vitro* digestion was important to identify previously unidentified amino acids (methionine and alanine) in fresh, cooked, and canned *A. bisporus* before digestion. Processing can lead to losses of soluble proteins and amino acids content in mushrooms However, processing did not affect the hydrolysis degree potential of the mushrooms.

In vitro digestion releases a much larger amount of essential amino acids than found in a free form in the mushrooms. Although A. *bisporus* has low protein content, *in vitro* digestion can release significant amounts of amino acids from mushrooms. The bioaccessibility data of spermidine in *A. bisporus*, can increase the value of this this food.

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DISCUSSÃO GERAL

As aminas bioativas e os aminoácidos livres são compostos que ocorrem naturalmente em uma variedade de alimentos exercem importantes funções no metabolismo humano como blocos de construção de proteínas (Marchini, 2016) ou estabilizadores do DNA and RNA (Kalač, 2014). Os cogumelos se classificam pelo elevado teor de espermidina, enquanto apresenta potencial para o desenvolvimento de outras aminas bioativas pela descarboxilação de aminoácidos livres. Dadaková et al. (2009) quantificaram em uma espécie selvagem (*Boletus erythropus*) o teor de 38,4 mg/100g (base úmida) de espermidina, o que demonstra o potencial ainda desconhecido desse alimento e abre dúvidas sobre quais fatores que podem influenciar o acúmulo de poliaminas em cogumelos.

Na Ásia e Europa, há uma cultura em torno dos cogumelos. Em que não somente são consumidos aqueles cogumelos cultivados e vendidos no mercado como aqueles cogumelos colhidos na natureza. No Brasil o consumo de cogumelos ainda é pequeno, contudo, tem aumentado, sendo o cogumelo *Agaricus bisporus* o mais consumido (ANPC, 2019). A comercialização de cogumelos *in natura* no Brasil é mais competitiva do que na forma em conserva, uma vez que os cogumelos frescos apresentam tempo de vida de prateleira curto e, portanto, não disputam mercado com países asiáticos como China que representa 80,1 % da produção mundial (ANPC, 2019; FAO, 2019).

A análise de aminas bioativas além de caracterizar o potencial biológico (principalmente pelas poliaminas), pode ser aplicada como critério higiênico sanitários no controle da qualidade dos cogumelos. A determinação de aminas bioativas simultânea a de aminoácidos livres pode representar um critério de identidade e qualidade mais abrangente, além de determinar o teor de ácido glutâmico que tem valorizado os cogumelos da espécie *Agaricus bisporus* pela característica desse aminoácido em compor o sabor umami de alimentos (Lee et al., 2009, Jawoska & Bernas, 2013, Rotola-Pulika 2019). Dessa forma, é importante ressaltar que estudos recentes sinalizam o consumo do micélio de cogumelo, por apresentar o desenvolvimento mais rápido, como uma alternativa futura ao consumo dos corpos de frutificação.

A fim de determinar pela primeira vez aminas bioativas e aminoácidos livres em cogumelos de forma simultânea em uma mesma corrida cromatográfica, foi desenvolvido um método analítico com base na extração ácida com ácido tricloroacético, derivatização pré-coluna com derivante carbamato de 6-aminoquinolil-N-hidroxisuquinimidil (Accq), separação em fase reversa por cromatografia ultra eficiência e detecção na região do ultravioleta (249 nm).

A cromatografia líequida de ultra eficiência é caracterizada pelo alto grau de resolução, capacidade de separação e tempo de análise curto. Dessa forma, não faz sentido um preparo de amostra dispendioso, trabalhoso e com tempo elevado. O preparo da amostra deve condizer com o nível de evolução da técnica analítica empregada. Por isso, as condições de análise de aminas bioativas e aminoácidos em cogumelos, foram otimizadas de forma que resultou em um preparo de amostra simples, rápido e econômico.

As condições cromatográficas inicialmente adotadas do método desenvolvido por Fiechter et al. (2013), tiveram que ser adaptadas em que para garantir a linearidade do método, a proporção de derivante por analito teve de ser aumentada, de forma que a quantidade do derivante estivesse em excesso com relação aos analitos. Ademais, de modo a estender a vida útil da coluna e garantir a robustez do método, as recomendações do manual do fabricante da coluna de fase reversa (CSH - C18 - 50 x 2.1 mm i.d., 1.7 µm). foram adotadas (*Waters Acquilty UPLC CSH columns Care and Use Manual*) em que foram reduzidas a vazão e a concentração do tampão acetato na fase móvel. Dessa forma o método apresentou-se simples e rápido de ser executado, com boa separação, resolução e foi validado de acordo com a norma 2002/657/EC.

Pelo fato dos cogumelos no Brasil serem consumidos principalmente cozidos e em conserva, o efeito do processamento sob o teor de aminas bioativas e aminoácidos foi estudado e comparado com o teor desses compostos nos cogumelos frescos. Inicialmente foi demonstrado que o teor de aminas e aminoácidos nos cogumelos podem variar conforme o lote de aquisição dos cogumelos e o teor desses analitos podem ser sensíveis ao processamento. Com relação ao teor de espermidina, os níveis dessa poliamina são mantidos após o processo de cozimento, contudo diminuem após o processo de conserva. Apesar dessa perda pelo processamento, o cogumelo em conserva pode ser classificado como alimento com alto teor de

poliamina (>1mg/100 ee4g) (Kalac, 2014). No tocante aos aminoácidos totais presentes nos cogumelos analisados, houve uma perda média de 39,7% no processo de cozimento e 64,6% no processo em conserva. A elevada redução de aminoácidos no processo em conserva também levará a uma maior perda de aminoácidos responsáveis pelo sabor dos cogumelos. Dessa forma, é necessário desenvolver futuramente técnicas mais brandas de processamento no intuito da manutenção dos aminoácidos livres, uma vez que são compostos importantes na composição do sabor dos cogumelos.

A adoção das análises de Componentes Principais e Agrupamentos Hierárquicos possibilitou a visualização do efeito do processamento e lote dos cogumelos em que a influência do processamento é maior que a influência do lote em relação ao teor total, uma vez que o processamento por si só leva a uma redução no teor de aminoácidos nos cogumelos. Após esse estudo o cogumelo *A. bisporus* produzido no Brasil, assim como em outros países (Lee et al., 2009, Jawoska & Bernas, 2013, Rotola-Pulika 2019), destaca-se pelo o elevado teor de ácido glutâmico que pode ser reduzido pelo processamento. A presença de aminoácidos livres precursores de aminas bioativas, representam o potencial de formação de aminas biogênicas. A ausência dessas no cogumelo analisado, representa a qualidade desse alimento e o valoriza como possível matéria prima para indústria farmacêutica e nutracêutica, visto que que não foram encontradas outras aminas bioativas com características neuro e vasoativas.

Com o objetivo de ter acesso às informações de bioacessibilidade das aminas bioativas e aminoácidos nos cogumelos, o ensaio de digestão *in vitro* foi conduzido no cogumelo fresco e processado, sendo avaliado a influência da fase gástrica e fase gástrica e intestinal e potencial para absorção do teor de aminas bioativas e aminoácidos. Além da digestão *in vitro*, para avaliar o potencial nutritivo desse alimento a digestibilidade proteica *in vitro* e o teor de proteína também foram determinados.

Com relação as aminas bioativas, das dez aminas analisadas, apenas a espermidina foi encontrada no cogumelo e nas frações relativas as etapas da digestão *in vitro.* Além disso o teor dessa poliamina não foi alterado ao longo do processo de digestão *in vitro*. Esse resultado quebra a hipótese que não só a espermidina, mas

outras aminas bioativas (putrescina, agmatina, cadaverina, tiramina, feniletilamina e histamina) poderiam estar na forma ligada nesses alimentos e serem detectadas ao longo do processo de digestão *in vitro*. Logo, é fato que o cogumelo *Agaricus bisporus*, não somente possuem alto teor espermidina como essa poliamina vai estar totalmente bioacessível desde a fase gástrica da digestão. Além da bioacessibilidade da espermidina. O estudo da digestão *in vitro* no cogumelo *Agaricus bisporus* permitiu acesso ao teor de aminoácidos acessíveis nesses cogumelos.

Apesar do processamento (principalmente na produção do cogumelo em conserva) reduzir o teor de aminoácidos livres no cogumelo, a digestibilidade das proteínas não foi afetada. Outro ponto importante, é que apesar dos cogumelos em sua composição centesimal apresentarem quantidade pequena de proteínas (< 2 g/ 100 g - base úmida), essas proteínas apresentaram bom grau de digestibilidade, sendo que ao final da digestão *in vitro* a quantidade de aminoácidos liberados dos cogumelos equivaleram a 45% do peso proteico nos cogumelos processados e 61.1% do peso proteico nos cogumelos. É válido ressaltar que esse percentual não considera o teor de di e tri peptídeos liberados durante o processo. Mesmo assim, é um percentual que demonstra a boa eficiência do processo de digestão *in vitro* na liberação de aminoácidos da matriz. Ademais, foi possível também avaliar a qualidade proteica desse alimento. Os valores da relação teor de cada aminoácido essencial liberados pelo processo de digestão *in vitro* por grama de proteína nos cogumelos fresco e processados (cozido e em conserva) estiveram adequados aos valores indicados pela FAO (2007) o que pode representar o valor da proteína encontrada nesses cogumelos. Dessa forma o sucesso do cogumelo Champignon de Paris (*Agaricus bisporus*) como alimento está condicionado aos elevados teores de teor ácido glutâmico (responsável pelo sabor umâmi) e sua digestibilidade. Os recentes dados sobre o teor de espermidina no cogumelo *A. bisporus* alimento podem agregar o valor desse alimento, como futura matéria prima para indústria de alimentos e farmacêutica.

CONCLUSÃO GERAL

Pelo presente objeto foi possível conhecer o estado da arte com relação ao perfil e teor de aminas bioativas e aminoácidos em cogumelos. Em que os cogumelos em geral são caracterizados pelo elevado teor de poliaminas e são estudados pelo seu potencial de formação de aminas biogênicas neuro e vaso ativas e aquelas que indicam a deterioração como cadaverina e putrescina. Apesar do elevado número de trabalhos que investigaram os aminoácidos livres em cogumelos, nunca foi levantada a relação de aminoácidos livres com o perfil e teor de aminas bioativas nos cogumelos.

Foi possível desenvolver uma metodologia analítica para a determinação simultânea de aminas bioativas, aminoácidos e íons amônio por UHPLC com detecção UV a qual foi otimizada e validada usando derivatização pré-coluna com carbamato de 6-aminoquinolil-N-hidroxisuccinimidil. Nesse método as condições de derivatização foram estabelecidas de forma a garantir respostas adequadas para todos os analitos e condições de operação condizentes com a técnica. Apesar de não ter sido possível estimar um modelo matemático para a extração de aminas e aminoácidos bioativos em cogumelos, foi possível estabelecer condições de extração brandas e rápidas.

O método foi validado de acordo com o regulamento 657/2002 de 16 de abril de 2002 da Comissão da Comunidade Europeia, em que a maioria dos analitos teve uma precisão satisfatória e os analitos apresentaram repetibilidade e reprodutibilidade satisfatórias. Quando aplicado à análise de amostras de cogumelos comerciais, o método foi rápido, fácil, preciso e confiável.

Com relação ao estudo do efeito do processamento, embora aminoácidos livres tenham sido identificados como precursores de aminas bioativas no cogumelo *Agaricus bisporus*, somente a espermidina foi identificada neste cogumelo. Isto confirma a qualidade sanitária higiênica dos cogumelos adquiridos. Além disso, o teor de poliaminas nos cogumelos *A. bisporus* pode variar dependendo dos diferentes lotes de cogumelos. Em relação ao processamento, o teor de espermidina é mantido após o cozimento, mas diminui significativamente após o processamento em conserva. O teor de aminoácidos livres nos cogumelos *A. bisporus* pode variar dependendo do lote e do processamento. O processo de cozimento e conserva levou à perda de

aminoácidos livres nos cogumelos, sendo maior no segundo processo, o que pode afetar o sabor desse alimento principalmente pela redução do teor de ácido glutâmico.

Com relação aos resultados do estudo sobre a bioacessibilidade das aminas bioativas e aminoácidos em cogumelos. A espermidina foi totalmente bioacessível nos cogumelos frescos, cozidos e em conserva. O estudo da digestão *in vitro* foi importante para identificar aminoácidos previamente não identificados antes da digestão, como a metionina e alanina. O processamento pode levar a perdas de proteínas solúveis e conteúdo de aminoácidos em cogumelos. A digestão *in vitro* é capaz de liberar uma quantidade muito maior de aminoácidos essenciais do que a encontrada de forma livre nos cogumelos.

Embora o cogumelo *A. bisporus* apresente baixo teor de proteína, a digestão *in vitro* é capaz de liberar quantidades significativas de aminoácidos dos cogumelos, o que pode corresponder a boa parte do peso proteico desse alimento. Os dados de bioacessibilidade da espermidina em *A. bisporus*, podem aumentar o valor deste alimento.

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