PATRÍCIA AMARAL SOUZA TETTE

Métodos analíticos para determinação da qualidade do mel por cromatografia líquida acoplada à espectrometria de massas

Faculdade de Farmácia da UFMG

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Tese apresentada ao Programa de Pós-Graduação em Ciência de Alimentos da Faculdade de Farmácia da Universidade Federal de Minas Gerais, como requisito parcial à obtenção do título de doutor.

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	UNIVERSIDADE FEDERAL DE MINAS GERAIS **PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA DE ALIMENTOS**	**PPGCA**

FOLHA DE APROVAÇÃO

MÉTODOS ANALÍTICOS PARA DETERMINAÇÃO DA QUALIDADE DO MEL POR CROMATOGRAFIA LÍQUIDA ACOPLADA À ESPECTROMETRIA DE MASSAS

PATRÍCIA AMARAL SOUZA TETTE

Tese submetida à Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em CIÊNCIA DE ALIMENTOS, como requisito para obtenção do grau de Doutora em CIÊNCIA DE ALIMENTOS, área de concentração CIÊNCIA DE ALIMENTOS.

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Belo Horizonte, 17 de outubro de 2016.

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LISTA DE ABREVIATURAS E SIGLAS

ABEMEL	Associação Brasileira de Exportadores de Mel
AED	atomic electron detector
ANOVA	análise de variância
APCI	atmospheric pressure chemical ionization
ASE	accelerated solvent extraction
CE	collision energy potentials
CME-UABE	coacervative microextraction ultrasound-assisted back extraction
CXP	collision exit potentials
DAD	detector de arranjo de diodos
DD	double derivatization
DLLME	dispersive liquid liquid microextraction
DP	declustering potential
dSPE	dispersive solid phase extraction
ECD	electron capture detector
ESI	electrospray ionization
ET	elevated temperature
FI	spectrofluorimetric detector
FPD	flame photometric detector
GC	gas chromatography
GC-MS	gas chromatography tandem mass spectrometry
HMF	Hidroximetilfurfural
HPLC	high performance liquid chromatography
HRMS	high-resolution mass spectrometry
HSE	Health and Safety Executive
HS-SPME	headspace solid phase microextraction
IBGE	Instituto Brasileiro de Geografia e Estatística
IL	ionic liquid
INMETRO	National Institute of Metrology, Quality and Technology
ISO	International Organization for Standardization
IT	ion trap
IT/MS	ion trap mass spectrometry
LC/DD/Fl	liquid chromatography double derivatization coupled with spectrofluorimetric detector
LC-MS/MS	liquid chromatography tandem mass spectrometry
LLE	liquid liquid extraction
LOD	limit of detection
LOQ	limit of quantification
LTP	low temperature purification
MAPA	Ministério da Agricultura Pecuária e Abastecimento
MEPS	microextraction by packed sorbent
MMC	matrix-matched calibration curves
MRL	maximum residue level
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MSPE	magnetic solid phase extraction
MU	Measurement uncertainty

nd	not detected
nf	not found
NPD	nitrogen phosphorus detector
OMC	Organização Mundial do Comércio
OPA	orto-ftalaldeído
PCRC	Plano de Controle de Resíduos em Carne
PCRL	Plano de Controle de Resíduos em Leite
PCRM	Plano de Controle de Resíduos em Mel
PCRP	Plano de Controle de Resíduos em Pescado
PNCRC	Plano Nacional de Controle de Resíduos e Contaminantes
PTR-MS	Espectrometria de massas baseada na reação de transferência de prótons
PSA	primary secondary amine
QuEChERS	quick, easy, cheap, effective, ruged and safe
RTWs	retention time windows
RSD	relative standard deviation
SBSE	stir bar sorptive extraction
SDME	single-drop microextraction
SLE	solid supported liquid liquid extraction
SPE	solid phase extraction
SPME	solid phase microextraction
TCA	ácido tricloroacético
TIC	total ion chromatogram
ToF	time of flight
UA	ultrasound-assisted
UE	União Europeia
UHPLC	Ultra performance liquid chromatography
UV	ultraviolet
WLS	weighted least squares

RESUMO

O mel, além de ser um adoçante natural e fonte de energia, apresenta efeito imunológico, antibacteriano, antiinflamatório, analgésico, sedativo e expectorante. A qualidade do mel pode ser influenciada por diversos fatores, incluindo a presença de contaminantes químicos, tais como os pesticidas. Ainda, a caracterização da origem botânica de méis monoflorais também é importante para a qualidade do produto, informação essa que garante autenticidade e aumenta o valor de mercado do mesmo. Este trabalho teve como objetivo desenvolver e validar métodos por LC-MS/MS para a análise multirresíduos de pesticidas em mel e também para certificar a autenticidade de mel de citros. O método validado para determinação de pesticidas utilizou a técnica QuEChERS modificada no preparo de amostras e UHPLC-MS/MS, e foi capaz de detectar e quantificar 116 pesticidas em amostras de mel. Os valores de recuperação variaram de 81,6 a 108,9%, o limite de detecção foi 0,005 mg/kg e os limites de quantificação foram de 0,01 e 0,025 mg/kg. Em geral, as amostras de mel analisadas apresentaram qualidade adequada quanto a presença de pesticidas. Apenas uma amostra, das 66 amostras de Minas Gerais analisadas, apresentou 0,029 mg/kg de triclorfon. Para a avaliação da autenticidade de mel de citros, foi proposto o uso de sinefrina, uma amina bioativa tipicamente encontrada em espécies de citros. O método por HPLC-MS/MS foi otimizado e validado e apresentou limites de detecção e de quantificação de 0,66 ng/g e 1 ng/g, respectivamente, e valores de recuperação entre 74,3 e 90,7%. A sinefrina foi detectada nas amostras que apresentaram alguma contribuição de florada de citros e, portanto, essa amina pode ser utilizada como um indicativo da presença de citros em amostras de méis. A origem do mel foi também determinada por análise qualitativa do pólen para confirmar a presença de citros.

Palavras-chave: Mel, Mel de laranjeira, Pesticidas, Sinefrina, LC/MS/MS.

ABSTRACT

Honey, besides being a natural sweetener and energy source, shows immunological effect, antibacterial, antiinflammatory, analgesic, sedative and expectorant activities. The quality of honey can be influenced by several factors, including the presence of chemical contaminants, such as pesticides. In adttition, the characterization of the botanical origin of monofloral honeys is important for product quality, information that ensures authenticity and increases its market value. The aim of this study was to develop and validate methods by LC-MS/MS for the multiresidue analysis of pesticides in honey and also to ensure citrus honey authenticity. The validated method for pesticides determination using a modified QuEChERS technique as sample preparation and UHPLC-MS/MS was suitable for detection and quantitation of 116 pesticides in honey. Recoveries ranged from 81.6 to 108.9%, the detection limit was 0.005 mg/kg and the limits of quantification were 0.01 and 0.025 mg/kg. In general, samples of honey showed appropriate quality in terms of pesticide residues. Only one of the 66 samples from Minas Gerais, had trichlorfon at 0.029 mg/kg. For authenticity of citrus honey, the use of synephrine, a bioactive amine tipically found in citrus species, was proposed. The developed HPLC-MS/MS method was optimized and validated and presented limits of detection and quantification of 0.66 ng/g and 1 ng/g, respectively, and recoveries between 74.3 and 90.7%. Synephrine was detected in samples with some contribution of citrus flowering and therefore this amine can be used as an indication of presence of citrus species in honey samples. The origin of honey was also determined by qualitative pollen analysis to confirm the presence of citrus.

Keywords: Honey, Orange honey, Pesticides, Synephrine, LC-MS/MS.

INTRODUÇÃO GERAL

O mel é um produto natural elaborado por abelhas a partir do néctar das flores (CAMPOS et al., 2003). O néctar coletado é transformado por meio de dois processos básicos, um físico - evaporação da água e outro químico - adição de enzimas (KOMATSU et al., 2002). A composição do mel consiste em proporções variáveis de água, açúcares, aminoácidos, sais minerais, óleo e enzimas especiais produzidas pelas abelhas (BOFFO et al., 2012).

Além de ser um adoçante natural e fonte de energia, o mel apresenta efeito imunológico, antibacteriano, antiinflamatório, analgésico, sedativo, expectorante e hiposensibilizador (SOUZA et al., 2004). Trata-se de um alimento complexo do ponto de vista biológico e também analítico, visto que a composição deste pode variar em função da respectiva origem floral e geográfica, assim como das condições edafoclimáticas do local de coleta do néctar (BASTOS, 1994; EVANGELISTA-RODRIGUES et al., 2005; FECHNER et al., 2016).

Apesar de todos os benefícios que este produto pode representar para a saúde humana, o mel não está livre de adulterações e contaminações por compostos químicos, dentre eles, antibióticos e pesticidas (BARGANSKA et al., 2011; KUJAWSKI & NAMIESNIK, 2011; LI et al., 2017). Diversos fatores podem alterar a qualidade do mel, fazendo com que o produto tenha características inadequadas e muitas vezes até inapropriadas para o consumo (DEBAYLE et al., 2008; TAHIR et al., 2016).

Estudos relacionados à qualidade do mel são necessários para garantir a segurança do consumidor e facilitar o comércio internacional. Para isto, as necessidades analíticas existentes nessa área devem ser conhecidas e alternativas devem ser propostas com vistas a auxiliarem na disponibilidade de produtos cada vez mais adequados, segundo parâmetros internacionalmente aceitos (SIMÕES, 2011; RIZELIO et al., 2012; SIDDIQUI et al., 2017).

A qualidade do mel está também associada ao tipo de florada. Méis provenientes de diferentes origens botânicas apresentam aromas e sabores diferenciados (BASTOS et al., 2002). Os méis monoflorais, especialmente o de laranjeira, tendem a ser mais valorizados comercialmente, devido ao sabor e

aroma característicos (ESCRICHE et al., 2011; VERZERA et al., 2014). Assim, estão mais sujeitos a adulterações e rotulagens fraudulentas (BOFFO et al., 2012).

Nos últimos anos, muitos métodos têm sido desenvolvidos para avaliar a qualidade e autenticidade dos méis produzidos no Brasil e em todo o mundo. Parâmetros físico-químicos, compostos voláteis, compostos antioxidantes, contaminantes orgânicos e inorgânicos têm sido extensivamente alvos de estudos (BILUCA et al., 2014; VERZERA et al., 2014; DOMINGUEZ et al., 2016; SERAGLIO et al., 2016; TAHIR et al., 2016). Portanto, métodos analíticos sensíveis necessitam ser desenvolvidos, sendo a escolha da técnica analítica, uma etapa de fundamental importância (SCHIRMER & MEISEL, 2006). Os avanços tecnológicos em espectrometria de massas visam atender, principalmente, os critérios de sensibilidade e seletividade (CHIARADIA et al., 2008). Neste sentido, a cromatografia líquida acoplada à detecção por espectrometria de massas sequencial (LC-MS/MS) tem demonstrado grande êxito em análises de diversos compostos em mel (KUJAWSKI & NAMIESNIK, 2011; TETTE et al., 2016a; SERAGLIO et al., 2016). Esta técnica fornece informações referentes ao tempo de retenção de cada composto, a obtenção de duas ou mais transições que permitem quantificar e confirmar o analito e elevada sensibilidade (MARTINS-JÚNIOR et al., 2006).

Vários fatores são determinantes para o estudo da qualidade do mel. Dentre eles, a possibilidade de adulteração e contaminação por pesticidas, as características dessa matriz que permitem utilizá-la como bioindicador de contaminação ambiental, e a escassez de estudos de desenvolvimento de métodos analíticos rápidos, eficientes e confiáveis para determinação de pesticidas nessa matriz. Além disso, a produção de mel no Brasil apresenta um grande potencial de crescimento e o desenvolvimento de alternativas que contribuam para garantir a autenticidade desse produto são necessárias. Dessa maneira, esse estudo teve como objetivo desenvolver e validar métodos analíticos por LC-MS/MS para determinação multi-resíduos de pesticidas em mel e também para garantir a autenticidade de mel de citros, por meio da utilização da sinefrina como biomarcador.

REVISÃO DA LITERATURA

MEL

1.1. Definição

O mel é um dos produtos da colméia mais utilizados, tanto *in natura* quanto em diversas formas industrializadas (KOMATSU et al., 2002). Conhecido desde a antiguidade, o mel sempre atraiu a atenção do homem, principalmente pelas características adoçantes, que o levaram a desenvolver técnicas cada vez mais aprimoradas, com o intuito de induzir uma maior produtividade das abelhas (ROSSI et al., 1999; BERA & ALMEIDA-MURADIAN, 2007).

Segundo a legislação brasileira, mel é definido como o produto alimentício produzido pelas abelhas melíferas, a partir do néctar das flores ou das secreções procedentes de partes vivas das plantas ou de secreções de insetos sugadores de plantas que ficam sobre as partes vivas de plantas, que as abelhas recolhem, transformam, combinam com substâncias específicas próprias, armazenam e deixam madurar nos favos da colméia (BRASIL, 2000).

Existem dois tipos principais de mel: (I) o mel produzido a partir do néctar das flores, e (II) o mel de melato produzido a partir de secreções de partes vivas de plantas ou de excreções de insetos sugadores (BERTELLI et al., 2010; SIDDIQUI et al., 2017).

A apicultura e a meliponicultura são duas áreas distintas de manejo de abelhas. A apicultura, muito popular, refere-se ao manejo racional de *Apis mellifera*, enquanto a meliponicultura refere ao manejo racional de abelhas sem ferrão, que têm apresentado amplo crescimento nos últimos anos (BILUCA et al., 2014). A regulamentação brasileira, baseada em legislações europeias, só atende às características do mel de *Apis mellifera*, não contemplando o mel das abelhas sem ferrão nativas do país, que apresentam diferenças em alguns parâmetros físico-químicos (ANACLETO et al., 2009).

O mel é uma matriz muito complexa, havendo, durante a respectiva elaboração, interferência de variáveis não controladas pelo homem, incluindo

clima, floração, presença de insetos sugadores e outros fatores. As abelhas, por sua vez, utilizam os recursos disponíveis como fonte de açúcar para elaborá-lo (CAMPOS et al., 2003). Tipos comuns de plantas usadas para produção de mel são eucaliptos, citros e flores silvestres (KOMATSU et al., 2002). O mel de origem floral pode ser produzido a partir do néctar de uma única espécie vegetal - denominado monofloral - ou de mais de uma espécie - polifloral (BASTOS et al., 2002). O mel monofloral pode ser produzido por meio da implantação de colméias onde as flores de uma determinada espécie de planta são predominantes (PROSSER & HEBERT, 2017). Geralmente, o monofloral é mais valorizado, com um flavor bem definido e preço de varejo consideravelmente mais elevado do que o de méis poliflorais (FALLICO et al., 2004; SILVA et al., 2008; VERZERA et al., 2014; ZHOU et al., 2014). Os reflorestamentos com eucalipto têm as flores intensamente visitadas por abelhas e constituem em eficientes fontes para a formação de méis apreciados pelos consumidores. Os méis de flores de laranjeira são também bastante procurados, não só por seu sabor suave, mas também por sua cor clara (KOMATSU et al., 2002).

1.2. Produção de mel por abelhas *Apis Mellifera*

A criação racional de abelhas é uma atividade que gera bons resultados econômicos, ecológicos e sociais. Essa atividade, desenvolvida ao longo dos anos por pequenos e médios produtores, vem despertando o interesse de muitas instituições do Brasil (EVANGELISTA-RODRIGUES et al., 2005). O mel é proveniente das abelhas e algumas vespas, porém, devido à domesticação antiga e por ser originária dos principais países consumidores, a abelha *Apis mellifera* é a espécie considerada como principal produtora do mel comumente utilizado para consumo humano, apesar da grande diversidade de espécies de abelhas existentes e que produzem mel de boa qualidade (ALVES et al., 2005). As abelhas utilizam parte desse mel para a própria alimentação e o restante é armazenado em quantidades consideráveis nos favos, para posterior abastecimento da prole num eventual período de escassez (BERA & ALMEIDA-MURADIAN, 2007).

As abelhas domésticas, *Apis mellifera*, são importantes para a produção comercial do mel e de produtos apícolas, como a geléia real e o pólen, mas também executam a tarefa vital de polinização das culturas agrícolas e das espécies nativas, sendo responsável por 80% da polinização (RISSATO et al., 2006; CHIARI et al., 2008; CHEN et al., 2016). Os polinizadores desempenham um importante papel funcional no ecossistema terrestre. Dados mostram que até 90% de toda espécie de planta com flor dependem da polinização por insetos (GARANTONAKIS et al., 2016). A cada dia, de 10.000 a 25.000 abelhas operárias fazem uma média de 10 viagens para explorar aproximadamente 7 km^2 nas áreas que cercam seu habitat, recolhendo o néctar, a água e o pólen das flores (RISSATO et al., 2006). A polinização constitui-se, atualmente, em um fator de produção fundamental na condução de muitas culturas agrícolas em todo mundo. Além do aumento no número de frutos vingados, a polinização também contribui para a melhoria da qualidade dos frutos, diminuição dos índices de malformação e ainda, uniformização do amadurecimento dos frutos, diminuindo as perdas na colheita (CHIARI et al., 2008).

1.3. A produção de mel no Brasil

A atividade apícola teve início no Brasil em 1839 com a introdução de abelhas da espécie *Apis Mellifera* no estado do Rio de Janeiro. Em 1956, a apicultura brasileira foi impulsionada por um acidente: a introdução da abelha africana (*Apis Mellifera Scutellata*), quando algumas abelhas escaparam do apiário experimental e passaram a se acasalar com as da raça européia, anteriormente trazidas pelos imigrantes, formando um híbrido natural chamado de abelha africanizada. Depois veio a profissionalização, e, desde então, o país tem se mostrado promissor para a atividade (SOUZA, 2011).

As abelhas africanizadas representam cerca de 90% das abelhas existentes no país. Essa contaminação possibilitou um grande salto em termos de produtividade (DE PAULA, 2008). A quantidade de mel produzido em 2014 foi de 38,47 mil toneladas, indicando um aumento de 8,8% em relação ao obtido no ano anterior. A Região Sul segue como a principal produtora, com 42,8% do total nacional, seguida pelas Regiões Nordeste (28,2%), Sudeste

(21,9%), Centro-Oeste (4,4%) e Norte (2,7%). O Estado do Rio Grande do Sul é o atual líder na produção de mel, com 5,99 mil toneladas, mas, apesar da colocação estadual, os Municípios de Botucatu (SP), Ribeira do Pombal (BA) e Içara (SC) se destacaram como os três maiores produtores (BRASIL, 2014). Na Tabela 1, estão apresentados os dados da produção nacional de mel em todas as unidades da federação e um comparativo dos anos de 2013 e 2014.

Tabela 1. Produção nacional de mel nas unidades federativas do Brasil nos anos de 2013 e 2014

Unidades da Federação	Quantidade (toneladas) por ano		Comparativo 2013/2014 (%)
	2013	2014	
Rio Grande do Sul	7.286	5.991	-17,8
Paraná	5.565	5.688	2,2
Santa Catarina	4.887	4.783	-2,1
Minas Gerais	3.308	3.821	15,5
São Paulo	3.224	3.481	8,0
Piauí	1.267	3.250	156,5
Bahia	2.058	3.148	53,0
Ceará	1.835	1.932	5,3
Maranhão	1.137	1.205	6,0
Mato Grosso do Sul	769	837	8,9
Espírito Santo	690	814	18,0
Pará	465	497	6,9
Mato Grosso	431	471	9,2
Pernambuco	503	393	-21,9
Goiás	344	355	3,3
Paraíba	160	320	100,0
Rio de Janeiro	373	313	-16,1
Rio Grande do Norte	331	312	-5,7
Rondônia	163	192	17,9
Roraima	133	187	40,4
Alagoas	146	186	27,4
Tocantins	118	119	1,0
Sergipe	97	101	4,0
Amazonas	37	38	2,6
Distrito Federal	20	20	-0,3
Amapá	9	10	7,6
Acre	8	9	14,2
Brasil	35.364	38.472	8,1

Fonte: Instituto Brasileiro de Geografia e Estística/2014 (BRASIL, 2014).

De acordo com a Associação Brasileira dos Exportadores de mel (ABEMEL), a média de consumo de mel por habitante no país é de apenas 60 gramas por ano, enquanto nos Estados Unidos da América e na Europa o consumo per capita ao ano gira em torno de 1,5 kg. Assim, o consumo desse alimento e a apicultura devem ser incentivados (ABEMEL, 2015). A apicultura é considerada uma das grandes opções para a agricultura familiar, por proporcionar o aumento da renda, por meio do aproveitamento da potencialidade natural do meio ambiente. É uma atividade promissora e que pode gerar trabalho e renda para um grande número de produtores. Isso significa a criação de novos empregos na zona rural e absorção da mão de obra familiar (SIMÕES, 2011).

1.4. Exportação de mel pelo Brasil

Até o ano 2000, o Brasil ocupava a 27º posição no ranking mundial de exportação de mel, com menos de 300 toneladas/ano. No ano de 2004, o Brasil alcançou a 5º posição entre os exportadores de mel, com mais de 20 mil toneladas/ano. O vertiginoso crescimento das exportações de mel brasileiro sofreu um grande impacto em 2006. No dia 17 de março de 2006, a União Europeia (UE) estabeleceu um embargo comercial, proibindo a exportação de mel brasileiro para o mercado europeu, sob alegação de descumprimento de exigências sanitárias de controle de resíduos. O mercado europeu representava, até então, o destino de 80% das exportações do mel brasileiro. A apicultura brasileira estava diante de um desafio estratégico de redirecionar sua produção para outros mercados. Apesar do embargo da União Europeia às exportações brasileiras, o Brasil conseguiu fechar o ano com incremento nas exportações de mel, redirecionando-as da Europa – sobretudo da Alemanha, até então o maior comprador do produto brasileiro – para os Estados Unidos (DE PAULA, 2008).

A exportação do mel brasileiro tem aumentado expressivamente nos últimos anos. Em termos de quantidade mundial de mel exportado, o Brasil subiu três posições no ranking de maior exportador, saindo da posição de 11º maior exportador em 2013, para 8º lugar no ranking das exportações em 2014

(Tabela 2). Foram exportados 25.317.263 kg e 22.205.764 kg nos anos de 2014 e 2015, respectivamente, sendo os Estados Unidos o principal destino das exportações. Em abril de 2016, a quantidade de mel exportado foi de 2.904.824 kg e 81,77% desse valor foram exportados para os Estados Unidos. Os estados brasileiros que se destacaram no mês de abril como maiores exportadores de mel para os Estados Unidos foram Piauí e São Paulo, com 51,24% do total. As exportações para os países europeus representaram 10,44% do total exportado em abril de 2016. Os estados brasileiros que se destacaram como exportadores de mel para Europa foram o Paraná e o Ceará, com 43,96% desse total (ABEMEL, 2016).

Tabela 2. Quantidade de mel exportado por país no ano de 2014

Posição no ranking mundial (2014)	País exportador	Volume exportado (toneladas)
1	China	129.824
2	Argentina	54.500
3	Vietnã	49.641
4	México	39.152
5	Ucrânia	36.336
6	Índia	26.976
7	Espanha	26.111
8	Brasil	25.317
9	Alemanha	22.547
10	Bélgica	20.006
11	Hungria	17.928
12	Tailândia	17.779
13	Polônia	13.719
14	Romênia	11.116
15	Uruguai	10.725
16	Bulgária	10.133
17	Nova Zelândia	9.504
18	Canadá	9.456
19	Itália	8.093
20	Chile	7.034
Exportação mundial		623.657

Fonte: Associação Brasileira dos Exportadores de Mel/2016 (BRASIL, 2016a).

1.5. Características físico-químicas e sensoriais

Quimicamente, o mel é composto por uma mistura de açúcares (WELKE et al., 2008) destacando-se principalmente a frutose (aproximadamente 38,5%) e a glicose (aproximadamente 31,0%) (BLASCO et al., 2011). Outros 22 açúcares, mais complexos, também podem estar presentes em pequenas concentrações, incluindo os dissacarídeos maltose, sacarose, maltulose, turanose, isomaltose, laminaribiose, nigerose, kojibiose, gentiobiose e β-trealose. Os trissacarídeos encontrados incluem maltotriose, erlose, melezitose, 1-kestose, isopanose, isomaltotriose, panose, e teanderose (BOGDANOV et al., 2004; SIDDIQUI et al., 2017). Entretanto, o percentual de açúcares varia em função da matéria-prima utilizada para sua elaboração, que pode constituir não apenas de néctar, mas também, de outras substâncias açucaradas, como suco de frutas em decomposição ou resultantes da transpiração das plantas (QUEIROZ et al., 2007).

Varios ácidos orgânicos também encontram-se presentes no mel, tais como os ácidos láctico, fórmico, butírico, tartárico, pirúvico, acético, cítrico, oxálico, succínico, málico, maléico, α-cetoglutárico, piroglutâmico, glicólico e glucônico. O ácido glucônico é o de ocorrência mais freqüente, produzido a partir de dextrose por ação da enzima glicose oxidase. A presença de enzimas no mel é uma característica única que o diferencia dos outros agentes edulcorantes. Estas enzimas são obtidas a partir das leveduras, néctar, pólen, abelha e micro-organismos. As enzimas mais importantes presentes no mel, além da glicose oxidase são catalase, fosfatase ácida, invertase e diastase (SIDDIQUI et al., 2017).

O mel também inclui outros constituintes em menores proporções como minerais, proteínas, aminoácidos, vitaminas, compostos fenólicos, flavonóides, pigmentos, aminas bioativas e outros componentes (FALLICO et al., 2004; FINOLA et al., 2007; KELLY et al., 2010; SILVA et al., 2016). No entanto, a composição do mel pode ser influenciada pela origem botânica, localização geográfica, condições climáticas, estádio de maturação, presença de insetos sugadores, tipos de abelhas produtoras, assim como pelo processamento e armazenamento deste produto. Por esta razão, podem apresentar consistência, cor, flavor e aroma diferentes (KOMATSU et al., 2002; SILVA et al., 2004;

DOWNEY et al., 2005; SILVA et al., 2008; WELKE et al., 2008). Além disso, a consistência e a textura também são influenciadas pela matéria-prima, em alguns casos, como consequência do teor e do tipo de proteína, que confere a alguns méis características gelatinosas ou, ainda, reduz a tensão superficial, tornando-o mais propenso a reter bolhas de ar e formar espuma (QUEIROZ et al., 2007).

O mel puro deve apresentar cor que poderá variar do amarelo ao amarelo avermelhado, com cheiro próprio, sabor doce e característico, aspecto líquido, denso, viscoso e translúcido (SILVA et al., 2004). A consistência do mel, característica conferida pela viscosidade, tem, como em outros alimentos, importância industrial e comercial. Industrialmente, maiores viscosidades implicam em maiores custos na centrifugação, decantação e misturas, ocasionando acréscimos nos tempos de operação e gastos energéticos. Do ponto de vista comercial, a qualidade do mel é associada, pelo consumidor, ao comportamento reológico, que identifica o mel puro como um produto de viscosidade mais elevada (QUEIROZ et al., 2007). Os diferentes comportamentos reológicos apresentados pelo mel de abelha também podem ser explicados por vários fatores tais como florada de origem, composição dos cristais presentes, bem como quantidade e tamanho desses cristais. Os méis variam significativamente conforme a espécie de abelha produtora, a começar pela característica sensorial conferida pela viscosidade (PEREIRA et al., 2003).

O aroma também é uma das características marcantes do mel muito apreciado pelos consumidores. Este decorre da presença de diferentes compostos voláteis. Mais de 600 compostos voláteis foram identificados e descritos em diferentes tipos florais. No entanto, acredita-se que existem muitos novos compostos voláteis a serem identificados em méis comuns e de diferentes floradas (TAHIR et al., 2016).

1.6. Características nutricionais e funcionais

O mel possui alto valor nutricional (BERA & ALMEIDA-MURADIAN, 2007). É um alimento de fácil digestão e assimilação, constituindo-se numa fonte de energia que contribui para o equilíbrio dos processos biológicos (KOMATSU et al., 2002). Devido às respectivas características de alta

absorção de carboidratos e alto valor calórico (303 kcal/100 g), o consumo de mel é interessante para pessoas de várias faixas etárias, especialmente para crianças com idades acima de um ano e atletas (BLASA et al., 2006).

O mel apresenta pequenas quantidades de vitaminas e as que estão presentes, com exceção da vitamina C, são essencialmente do complexo B, devido a essas serem características dos grãos de pólen em suspensão. As vitaminas encontradas no mel incluem tiamina (B1), riboflavina (B2), ácido nicotínico (B3), o ácido pantoténico (B5), piridoxina (B6), biotina (B8 ou H) e ácido fólico (B9). Essas vitaminas presentes no mel são preservadas devido ao baixo pH (BONTÉ & DESMOULIÈRE, 2013).

Os minerais estão presentes no mel em proporções de apenas 0,1% e são mais abundantes em méis escuros. Potássio, cálcio, sódio, magnésio, cobre, manganês, cloro, enxofre, silício, ferro e mais de trinta oligoelementos são encontrados no mel. O conteúdo desses depende das plantas visitadas pelas abelhas e do tipo de solo em que as plantas foram cultivadas (BONTÉ & DESMOULIÈRE, 2013).

Além de ser apreciado pelo sabor característico e considerável valor nutritivo (BERA & ALMEIDA-MURADIAN, 2007), o homem também tem utilizado o mel como medicamento, devido às propriedades de prevenção de doenças, antimicrobianas e anti-sépticas que este apresenta (CORTOPASSI-LAURINO & GELLI, 1991; SILVA et al., 2008; IURLINA et al., 2009). Vários compostos presentes no mel estão sendo estudados por apresentar atividade antioxidante, incluindo os ácidos vanílico, gálico, caféico, siríngico, cumárico, ferúlico e clorogênico, e kaempferol, galangina, triacetina, luteolina, miricetina, quercetina, crisina, pinobanksina, vitamina C, catalase, pinocembrina, entre outros (BLASA et al., 2006; IURLINA et al., 2009; PYRZYNSKA & BIESAGA, 2009; ISLA et al., 2011). No entanto, dentre os principais componentes funcionais do mel estão os flavonóides. Estes podem contribuir significativamente para a atividade antioxidante total do mel, trazendo efeitos benéficos para a saúde humana, eliminando radicais livres e inibindo a oxidação lipídica (SILVA et al., 2016). A capacidade antioxidante do mel depende da fonte floral utilizada pelas abelhas para recolher o néctar, bem como de fatores sazonais e ambientais (YAO et al., 2003; ESCRICHE et al., 2011).

As propriedades curativas do mel têm sido conhecidas na medicina tradicional desde a antiguidade. Durante o século passado, o mel foi submetido a inúmeras investigações clínicas e laboratoriais, o que demonstrou mais uma vez os seus efeitos benéficos como anti-séptico, propriedades de cicatrização quando aplicado topicamente e inibição de estirpes de *Staphylococcus* resistentes à meticilina, entre outras bactérias (ZUMLA & LULAT, 1989; KUJAWSKI & NAMIESNIK, 2011). A atividade antimicrobiana dos méis tem sido atribuída a ação individual ou sinérgica de fatores como pH, conteúdo de açúcares, níveis de peróxido de hidrogênio e a presença de compostos fenólicos, incluindo ácidos fenólicos e flavonóides (WESTON, 2000; IURLINA et al., 2009; ISLA et al., 2011).

1.7. Avaliação da qualidade do mel

1.7.1. Determinação de parâmetros físico-químicos

O Brasil possui reservas florais que podem proporcionar milhares de toneladas de mel de primeira qualidade aceito pelos mercados mais exigentes do mundo (EVANGELISTA-RODRIGUES et al., 2005). Porém, no Brasil, a produção ainda não é suficiente para atender à demanda, ocasionando uma maior valorização do produto e fazendo com que este seja alvo de adulterações (ROSSI et al.,1999; BERA & ALMEIDA-MURADIAN, 2007).

O Ministério da Agricultura, Pecuária e Abastecimento (MAPA) publicou regulamento técnico de identidade e qualidade do mel, por meio da Instrução Normativa nº 11, de 20 de outubro de 2000 (BRASIL, 2000). Esta tem como objetivo estabelecer a identidade e os requisitos mínimos de qualidade para o mel destinado ao consumo humano direto.

O mel é 100% natural e nada deve ser extraído ou adicionado a ele (BOFFO et al., 2012). O tipo de adulteração mais comum é aquela com ingredientes de baixo valor comercial, tais como açúcar comercial, glicose, melado e solução de açúcar invertido (ROSSI et al.,1999; BERA & ALMEIDA-MURADIAN, 2007). Xarope de milho rico em frutose, xarope de beterraba e xarope de maltose são os mais utilizados (LI et al., 2017). Considerando que pode ocorrer a deliberada adição desses xaropes ao produto, a determinação

da frutose, glicose e sacarose tem sido usada para descrever a qualidade e autenticidade do mel (DOMINGUEZ et al., 2016).

Alguns parâmetros como umidade, acidez, condutividade elétrica, atividade diastásica e teor de hidroximetilfurfural (HMF) também são utilizados na avaliação da qualidade do mel (CAMPOS et al., 2003; BOGDANOV et al., 2004; FALLICO et al., 2004). Esses parâmetros estão relacionados com a qualidade nutricional, a granulação, o sabor e a textura (SIDDIQUI et al., 2017). O conteúdo de umidade do mel está relacionado com a sua maturidade. Baixos teores de umidade auxiliam na estabilidade do produto durante o período de estocagem, enquanto altos teores podem indicar a adulteração por adição de água (ANKLAM, 1998). A condutividade elétrica depende do conteúdo mineral, ácidos orgânicos, proteínas, alguns açúcares complexos e polióis. Quanto maior o teor de íons e ácidos orgânicos, maior a condutividade (TERRAB et al., 2003). Altos valores de acidez livre podem indicar que o mel foi contaminado por leveduras e os açúcares foram fermentados (ANKLAM, 1998). Além disso, a atividade diastásica e o conteúdo de HMF são reconhecidos mundialmente como indicadores de frescor e sobreaquecimento do mel, sendo que uma amostra de qualidade apresenta alta atividade diastásica e baixo conteúdo de HMF, isso porque o aquecimento excessivo e o armazenamento por longos períodos contribuem para a diminuição da atividade diastásica e também levam à formação de HMF (ANKLAM, 1998; FALLICO et al., 2004; RIZELIO et al., 2012).

Geralmente, as alterações na composição química do mel ocorrem durante o processamento e no período de armazenamento. Dessa forma, esses parâmetros necessitam ser avaliados de forma constante durante o armazenamento para assegurar a manutenção das características e estabilidade de compostos químicos presentes no mel (SILVA et al., 2016).

1.7.2. Detecção de pesticidas

O mel deve ser livre de qualquer contaminação química ou biológica para ser seguro para o consumo humano (PINHO et al., 2010). No entanto, este não está livre de contaminações por pesticidas (KUJAWSKI & NAMIESNIK, 2011; TETTE et al., 2016b). Os resíduos destes compostos

podem estar presentes no mel, expondo os consumidores aos efeitos adversos aos seres humanos (CALDAS & SOUZA, 2000; DEBAYLE et al., 2008). O comércio internacional do mel também é prejudicado devido às barreiras não alfandegárias impostas pelos importadores (PERES et al., 2007).

Pesticidas podem ser utilizados no tratamento de pragas que surgem na colméia, durante a colheita do mel e resultar em possível rota de contaminação (KUJAWSKI & NAMIESNIK, 2011). Estes resíduos podem ser provenientes do tratamento das colmeias com acaricidas no controle de *Varroa jacobsonie* e *Ascosphera apis* (KUJAWSKI & NAMIESNIK, 2008; MUKHERJEE, 2009; BLASCO et al., 2011). Contaminações indiretas do mel também podem ocorrer durante a aplicação de pesticidas na agricultura, por meio da contaminação do solo, do ar, da água e das flores das quais as abelhas coletam o néctar para a produção de mel (KUJAWSKI & NAMIESNIK, 2008; MUKHERJEE, 2009; PINHO et al., 2010; BLASCO et al., 2011). Estes contaminantes podem ser levados para a colméia nos corpos das abelhas ou com as forrageiras e entrar em contato com o mel (KUJAWSKI & NAMIESNIK, 2011). Altas concentrações de resíduos de pesticidas conduzem a alta taxa de mortalidade de abelhas e o mel produzido é impróprio para consumo humano (PINHO et al., 2010).

O Plano Nacional de Controle de Resíduos em Produtos de Origem Animal (PNCR) do Ministério da Agricultura, Pecuária e Abastecimento (MAPA) foi instituído pela Portaria Ministerial nº 51, de 6 de maio de 1986, adequado pela Portaria Ministerial nº 527, de 15 de agosto de 1995, e modificado pela Instrução Normativa nº 42, de 20 de dezembro de 1999. Este é constituído de Programas Setoriais para carne – PCRC, mel – PCRM, leite – PCRL, e pescado – PCRP. O PNCR tem como um dos seus objetivos melhorar a produtividade e qualidade dos alimentos de origem animal disponibilizados à população brasileira e proporcionar à nação condições de se adequar, do ponto de vista sanitário, às regras do comércio internacional de alimentos preconizadas pela Organização Mundial do Comércio (OMC) e órgãos auxiliares. O Plano concentra todo esforço governamental, no sentido de ofertar aos consumidores alimentos seguros e competitivos (BRASIL, 1999).

Na Instrução Normativa nº 13, de 15 de julho de 2015, está contido o Subprograma de Monitoramento e Subprograma Exploratório do PNCR de

2015 para as cadeias de carnes (bovina, suína, caprina, ovina, equina, de coelho, de aves e de avestruz), de leite, pescado, mel e ovos. Para o mel, estão previstos o monitoramento dos seguintes grupos: antimicrobianos, compostos halogenados e organoclorados, carbamatos, piretróides, organofosforados e contaminantes inorgâncios (BRASIL, 2015). Como pode ser notado, essa instrução prevê um número reduzido de classes de pesticidas a serem monitoradas. Além disso, apesar de alguns estudos realizados no Brasil contemplarem algumas classes a mais do que o previsto no PNCRC, ainda não há estudos que possam ser considerados abrangentes (Tabela 3).

Tabela 3. Ocorrência e níveis de pesticidas em amostras de méis comerciais do Brasil

Classes de pesticidas	Amostras		Faixa (µg/kg)	Referência
	Analisadas	Positivas (%)		
Organofosforados, piretróides, organoclorados, strobina, triazol, cloronitrila, pirazol e dinitroanilinas	4	100	nd - 90	ORSO et al., 2014
Organoclorados, organofosforados, carbamatos, piretróides, dicarboximida e outros não classificados	20	0	nd	SALAMI et al., 2013
Clorofenoxi, feniluréia e fenilpirazol	2	0	nd	SAMPAIO et al., 2012
Amidina, benzimidazol, organofosforados, fenilpirazol, piretróides, neonicotinóides, triazol	3	0	nd	TOMASINI et al., 2012
Organofosforados Piretróides	11	18	nd – 0,22	PINHO et al., 2010

nd= não detectado.

A técnica analítica utilizada para determinação de pesticidas em mel desempenha um papel fundamental na qualidade dos resultados (KUJAWSKI et al., 2014). Um método multiresíduo capaz de detectar e quantificar os pesticidas, em um período de tempo relativamente curto, compreendendo etapas mínimas de extração e purificação, é crucial para um programa de monitoramento eficiente (RISSATO et al., 2006). Nesse sentido, a cromatografia líquida acoplada à detecção por espectrometria de massas

sequencial (LC-MS/MS) tem sido a técnica de preferência para a análise multiresíduos de pesticidas em mel, devido às características de seletividade e sensibilidade desta técnica.

1.7.3. Avaliação da autenticidade do mel

Nos últimos anos, além do interesse na segurança, consumidores, produtores e autoridades reguladoras também estão interessados na autenticidade do mel, por meio da rotulagem, da origem botânica e da rastreabilidade. O critério de origem geográfica é um parâmetro de qualidade para produzir mel certificado com denominação de origem. Assim, a classificação regional de méis de acordo com as zonas de produção dos mesmos favorece a valorização do produto (SILVANO et al., 2014; FECHNER et al., 2016). Além disso, alguns tipos de méis apresentam valor comercial mais elevado que outros, devido às características específicas, especialmente em relação à florada. Portanto, a fim de evitar a rotulagem fraudulenta, meios de diferenciação entre os tipos de méis necessitam ser desenvolvidos (BOFFO et al., 2012; TAHIR et al., 2016).

Dentre os méis comercializados no Brasil, os de flores silvestres se destacam por sua maior disponibilidade no mercado, pois este pasto apícola é oferecido às abelhas durante o ano todo. Os reflorestamentos com eucalipto, que tem as flores intensamente visitadas por abelhas, também se constituem em eficientes fontes para a formação de méis apreciados pelos consumidores e, finalmente, os méis de flores de laranjeira são bastante procurados, não só por seu sabor suave, mas também por sua cor clara (KOMATSU et al., 2002).

A classificação botânica dos méis é realizada por meio de identificação e quantificação da porcentagem de polens presentes na amostra por análise microscópica, conhecida como melissopalinologia (BELAY et al., 2015). No entanto, a análise melissopalinológica apresenta algumas desvantagens, tal como a necessidade de um analista com grande experiência na identificação da morfologia dos polens. Especialmente para méis de citros, essa análise é considerada de pouco valor, porque os polens nesses tipos de méis estão presentes em quantidades menos significativas (RODRIGUEZ et al., 2010; ESCRICHE et al., 2011).

Em virtude das limitações existentes na análise melissopalinológica, estão sendo desenvolvidos vários métodos químicos complementares para a determinação da autenticidade de méis. Tahir et al. (2016) estudaram os perfis de aroma de seis méis de diferentes origens botânicas por meio de sensores colorimétricos e cromatografia a gás associada à espectrometria de massas (GC-MS). Cinquenta e oito compostos aromáticos foram identificados e 20 compostos mais abundantes foram utilizados para caracterizar os méis com o auxílio de análise multivariada. Kus e Ruth (2015) desenvolveram modelos de classificação quimiométrica utilizando HPLC-DAD e espectrometria de massas baseada na reação de transferência de prótons (PTR-MS). Impressões digitais das amostras proporcionaram a identificação de méis de diferentes origens botânicas, em taxas que variaram de 67 a 100%. O método desenvolvido por Verzera et al. (2014) foi baseado na razão enantiomérica de compostos voláteis quirais derivados de plantas visitadas por abelhas e que podem estar presentes no mel e flores de laranjeira. A fração volátil das amostras foi estudada, e o composto linalol e seus óxidos, mostraram bom potencial para identificação dessas amostras. Flavonóides e compostos fenólicos foram também utilizados com o objetivo de diferenciar méis de laranja e limão (ESCRICHE et al., 2011). Nesse estudo, os autores identificaram que méis de limão apresentam duas vezes mais flavonóides e ácidos fenólicos quando comparado ao mel de laranja. Análises multivariadas também foram conduzidas para diferenciar os dois tipos de méis, e alguns compostos foram apontados como chave nessa identificação.

Apesar dos esforços sendo realizados no sentido de desenvolver métodos químicos para avaliar a autencidade de méis, poucos estudos estão disponíveis na literatura especificamente para mel de laranjeira (VERZERA et al., 2014). Além disso, grande parte dos métodos desenvolvidos até o presente momento envolve um grande número de compostos, são trabalhosos e demorados. Portanto, existe a necessidade do desenvolvimento de técnicas práticas e com resultados satisfatórios (BOFFO et al., 2012).

Embora inúmeros parâmetros de identidade e qualidade sejam analisados constantemente em amostras de mel, a autenticidade é um importante desafio para assegurar uma melhor qualidade do produto para comercialização (SILVA et al., 2016; SIDDIQUI et al., 2017).

OBJETIVOS

O objetivo geral deste trabalho foi desenvolver métodos analíticos de extração, separação e detecção para a determinação de multi-resíduos de pesticidas em mel e de marcador de autenticidade de mel de laranja, ambos por meio de cromatografia líquida acoplada à espectrometria de massas sequencial (LC-MS/MS).

Os objetivos específicos foram:

- ✓ fazer uma revisão extensiva sobre os métodos analíticos cromatográficos utilizados para análise de pesticidas em mel;

- ✓ desenvolver e validar método analítico para a extração, separação e quantificação de pesticidas em mel por LC-MS/MS, e aplicar o método desenvolvido na análise de amostras de mel de diferentes regiões do Brasil;

- ✓ desenvolver e validar método analítico para a extração, separação e quantificação de sinefrina em mel por LC-MS/MS. Aplicar o método proposto na análise de amostras de mel de laranjeira e de diferentes floradas.

CAPÍTULO I - PESTICIDES IN HONEY: A REVIEW ON CHROMATOGRAPHIC ANALYTICAL METHODS

ABSTRACT

Honey is a product of high consumption due to its nutritional and antimicrobial properties. However, residues of pesticides, used in plagues' treatment in the hive or in crop fields in the neighborhoods, can compromise its quality. Therefore, determination of these contaminants in honey is essential, since the use of pesticides has increased significantly in recent decades because of the growing demand for food production. Furthermore, pesticides in honey can be an indicator of environmental contamination. As the concentration of these compounds in honey is usually at trace levels and several pesticides can be used simultaneously, the use of highly sensitive and selective techniques is required. In this context, miniaturized sample preparation approaches and liquid or gas chromatography coupled to tandem mass spectrometry became the most important techniques in this field of analysis. In this review we present and discuss the recent studies dealing with pesticide determination in honey, focusing on sample preparation and separation/detection methods as well as application of the developed methods worldwide. Furthermore, trends and future perspectives are presented.

Keywords: Honey; Pesticides**;** Multiresidue method**;** Sample preparation**;** LC-MS/MS; GC-MS

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1. Introduction

The use of pesticides has increased significantly during the last decades (NEUFELD et al., 2000; VARSAMIS et al., 2008; LLORENT-MARTINEZ et al., 2011). Although the use of these compounds brings benefits to agriculture, many of them reach a distinct destination than the target species and could contaminate soil, water and food. Since some pesticides are carcinogenic and some can cause dysfunctions in the nervous and reproductive systems, even at low concentrations, they can be extremely harmful to human health (NEUFELD et al., 2000; VARSAMIS et al., 2008; SHARMA et al., 2010). Thus, the risks to food safety due to the use of these compounds are constant motives to world concern (PANG et al., 2006a; KOLBERG et al., 2011).

The monitoring of pesticides in honey is necessary to warrant consumers' safety. Furthermore, the control of pesticides in honey can provide information about the use of pesticides in crop fields and in the neighborhoods (RISSATO et al., 2006). According to Rissato et al. (2006) bees and honey can be used as biomarkers for monitoring environmental contamination. Thus, analytical methods for the routine determination of pesticides in honey are needed. Today's analytical challenge is the use of multiresidue methods capable of analyzing several pesticides simultaneously; with high sensitivity and specificity; with minimal use and disposal of solvents which can be detrimental to human and environmental health; and fast (WIEST et al., 2011; GOMEZ-PEREZ et al., 2012).

The determination of pesticide in food requires sample preparation, separation and quantification. Furthermore, the performance of the method must be investigated to demonstrate its fitness for the purpose. Due to the low concentration of pesticides in the sample, the distinct chemical properties and the matrices complexity, sample extraction, purification and concentration are needed (PRESTES et al., 2009). Most of the sample preparation procedures are carried out by conventional techniques, such as liquid-liquid extraction (LLE); however, they have the disadvantages of being expensive and using large amounts of organic solvents, which are generally toxic for the technician and can contaminate the environment. These limitations have led to the

development of new techniques which are convenient, consume less organic solvents and have the ability to detect analytes in very low concentrations. In recent years, efforts in the field of analytical chemistry focused on the miniaturization of sample preparation associated with increased selectivity and sensitivity (MELWANKI & FUH, 2008).

However, most of these efforts are far from being ideal. Miniaturized extraction techniques, developed recently, have been applied and optimized for the extraction of pesticides from honey, in order to solve the problems of conventional methods (DU et al., 2013; SALAMI et al., 2013; KUJAWSKI et al., 2014). However, they still have limitations on application, quickness, sensitivity and reliability of the results.

Besides the extraction and purification procedures, the choice of the separation/detection approach is of fundamental importance. Technological advances in mass spectrometry have achieved the need for sensitivity and selectivity (CHIARADIA et al., 2008). Liquid chromatography coupled with tandem mass spectrometric detection (LC-MS/MS) and gas chromatography coupled with tandem mass spectrometric detection (GC-MS/MS) have shown great success in multiresidue analysis of antibiotics and pesticides in honey (DEBAYLE et al., 2008; BARGANSKA et al., 2013; JOVANOV et al., 2013; PANSERI et al., 2014). These techniques provide information regarding the retention time of each compound and allows gathering of two or more transitions to quantify and confirm the identity of the analyte. They also present high sensitivity, consistent with the Maximum Residue Levels (MRLs) established by the international legislation (MARTINS JÚNIOR et al., 2006).

In 2007, Rial-Otero et al. (2007) published a review on methods employed for pesticide analysis in honey. They also presented the trends they expected to become reality in the following years. After this review several innovative techniques of sample preparation, separation and detection were developed and employed for determination of pesticides in honey. In this context, this review aims to present and discuss the studies published in the period between 2008 and 2015 dealing with pesticide determination in honey. Special focus was given on sample preparation and separation/detection methods as well as application of the developed methods worldwide.

2. Honey

The Codex Alimentarius defines honey as the natural sweet substance produced by honey bees from the nectar of plants or from secretions of living parts of plants or excretions of plant sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in the honey comb to ripen and mature (WHO, 2015).

Honey is composed of a mixture of sugars (WELKE et al., 2008), mainly fructose (~38.5%) and glucose (~31.0%) but also maltose, sucrose and other complex carbohydrates (BLASCO et al., 2011). However, the percentage of sugars varies depending on the raw material used for its production (QUEIROZ et al., 2007). It also contains other components in minor proportions, such as minerals (calcium, copper, iron, magnesium, phosphorus, potassium), proteins, amino acids, vitamins, flavonoids, pigments, several organic acids, and compounds with antioxidant properties including chrysin, pinobanksin, vitamin C, catalase and pinocembrine (FALLICO et al., 2004; FINOLA et al., 2007; KUJAWSKI & NAMIESNIK, 2008; SILVA et al., 2008; BLASCO et al., 2011). The physicochemical evaluation of honey is important for its characterization and to ensure the quality of the product in the market (SERRANO et al., 2004; WELKE et al., 2008).

Besides being appreciated for the characteristic flavor and nutritional value (BERA & ALMEIDA-MURADIAN, 2007), humans have also used honey due to its antimicrobial and antiseptic properties, and as a preservative in fruit and grains (CORTOPASSI-LAURINO & GELLI, 1991; SILVA et al., 2008). The healing properties of honey have been known in medicine since ancient times. During the last century, honey was subjected to numerous clinical and laboratorial investigations, which confirmed their medical benefits as antimicrobial, especially against *Staphylococcus* strains which are resistant to methicillin among other bacteria (ZUMLA & LULAT,1989; KUJAWSKI & NAMIESNIK, 2011).

2.1. Pesticides in honey

The constant growth of the world's population has demanded increased food production. However, annual losses due to plagues on agriculture are about 1 billion ton around the world, with a decrease of 20% to 30% on production (PANG et al., 2006b). Therefore, to overcome this problem, the chemical industries are looking for new substances with activity against plagues and other biological threats (CIESLIK et al., 2011). Currently, there are more than 100 pesticides registered in the European Union's market (JEONG et al., 2012).

Pesticides (herbicides, fungicides, insecticides or acaricides) have an important role in the development of agriculture, warranting increased agricultural production (PANG et al., 2006a; PEREZ-FERNANDEZ et al., 2010; KOLBERG et al., 2011). In this context, the use of pesticides has increased significantly in recent decades (VARSAMIS et al., 2008; LLORENT-MARTINEZ et al., 2011). Despite the benefits of the use of these chemical compounds, they are also responsible for contamination of soil, water and food (BLASCO et al., 2011), which results in environmental accumulation of contaminants which can be introduced into the human food chain (CIESLIK et al., 2011).

According to Llorent-Martínez et al. (2011), more than 98% of the insecticides and 95% of the herbicides sprayed do not reach their final destination, which are the target species. Pesticides are frequently found in water, soil, atmosphere and agricultural products and they can represent an environmental threat (VARSAMIS et al., 2008; LLORENT-MARTINEZ et al., 2011). Even at low concentrations, contaminants can cause adverse effects to humans, plants, animals and also to the ecosystems as some of them are carcinogenic, and others can cause dysfunctions in the nervous system (NEUFELD et al., 2000; VARSAMIS et al., 2008; SHARMA et al., 2010). Therefore, the presence of pesticides in foods and in the environment has been a serious worldwide health and safety concern (PANG et al., 2006a; KOLBERG et al., 2011) and the demand for the detection of chemicals which can pose environmental risks has increased in the last years.

Honey must be free of any chemical or biological contamination to be safe for human's consumption (PINHO et al., 2010). However, pesticides can

be used in plagues' treatment in the hive during honey harvesting, resulting in a possible contamination route (KUJAWSKI & NAMIESNIK, 2011). Furthermore, the hive can be treated with acaricides for the control of *Varroa jacobsoni* and *Ascosphaera apis* (BLASCO et al., 2011). The most commonly used pesticides are amitraz, cymiazole, bromopropylate, coumaphos, flumethrin, fluvalinate, imidacloprid and fipronil (PINHO et al., 2010).

Indirect contamination of honey can also occur during the application of pesticides in agriculture, through soil, air, water, and flowers where bees visit and collect nectar to produce the honey (PINHO et al., 2010). These chemicals can be carried to the hive by the bees' bodies or by forage and contaminate the honey (KUJAWSKI & NAMIESNIK, 2011). High pesticides' concentrations can lead to a high mortality rate of bees and the production of inappropriate honey for human consumption (PINHO et al., 2010).

Table 1 shows the maximum residual limits (MRLs) for pesticides in honey adopted by Brazil (29 pesticides) (BRASIL, 2015), European Union (20 out of 279 pesticides) (EU, 2015), United Kingdom (21 out of 327 pesticides) (HSE, 2015), United States (4 pesticides) (US, 2015) and Australia (5 pesticides) (FSANZ, 2015). It is interesting to observe that legislation from European Union and the United Kingdom presents a comprehensive list of MRLs for pesticides in honey, but only 18 and 24, respectively, are similar to the pesticides listed by the Brazilian legislation. On the other hand, only four and five pesticides in honey were found in the legislation from the United States and Australia, respectively.

Programs for monitoring pesticides in honey are usually focused on the determination of acaricides, which are used to control *Varroa jacobsoni* (RUFFINENGO et al., 2005; SATTA et al.,2005). A few studies have focused on pesticides used to protect plantations and those introduced in the hive by the bees (FAUCON et al., 2005; RISSATO et al., 2006).

Table 1. Maximum Residual Limits (MRLs) of pesticides in honey according to the legislations from Brazil, Europe (EU), United States, United Kingdom and Australia

Class	Compounds	Maximum residual limits (MRLs) in honey (µg/kg)				
		Brazil	European Union	United States	United Kingdon	Australia
Halogenated and Organochlorine	Aldrin*	10	10[d]	nf	10[d]	nf
	α-Endosulfan*	10	10[c]	nf	10[c]	nf
	4,4-DDE*	10	50[b]	nf	50[b]	nf
	4,4-DDD*	10	50[b]	nf	50[b]	nf
	4,4 DDT*	10	50[b]	nf	50[b]	nf
	Dodecachlor*	10	nf	nf	nf	nf
	Endrin*	10	10	nf	10	nf
	Tetradifon*	20	50	nf	50	nf
	Vinclozolin*	20	50	nf	50[e]	nf
	Heptachlor*	10	10[f]	nf	10[f]	nf
	α-HCH*	10	nf	nf	nf	nf
	β-HCH*	10	nf	nf	nf	nf
	Ɣ-HCH*	10	nf	nf	nf	nf
Carbamates	Carbofuran*	50	10[h]	nf	10[i]	nf
	Carbaryl*	20	50	nf	50	nf
	Captan*	50	50	nf	50	nf
Pyrethroids	Fluvalinate	nf	50	20	nf	10
	Flumethrin	nf	nf	nf	nf	5
	Permethrin*	20	nf	nf	nf	nf
	Cyfluthrin*	20	50[g]	nf	50[g]	nf
	Fenpropathrin*	10	nf	nf	nf	nf
	Deltamethrin*	20	30[j]	nf	30[j]	nf
	Amitraz*	200	nf	200	nf	nf
Organophosphates	Coumaphos	nf	nf	150	nf	nf
	Chlorpyrifos*	20	nf	nf	nf	nf
	Dimethoate*	20	nf	nf	nf	nf
	Disulfoton*	10	10[l]	nf	10[l]	nf
	Pirimiphos methyl*	50	nf	nf	nf	nf
	Parathion*	20	nf	nf	nf	nf
	Fenamiphos*	10	10[m]	nf	10[m]	nf
	Terbufos*	10	nf	nf	10	nf
	Profenofos*	20	50	nf	50	nf
Pyrazole	Fenpyroximate	nf	50	100	50	nf
	Fipronil	nf	5	nf	5[a]	10
Others	Oxytetracycline	nf	nf	nf	nf	300
	Phosphine	nf	nf	nf	10	10

* Pesticides for which Brazil established limits according to the National Control Plan for Residues and Contaminants (PNCRC) of the Ministry of Agriculture, Livestock and Food Supply (BRASIL, 2015); [a]Fipronil = sum fipronil + sulfonemetabolite (MB46136) expressed as fipronil; [b]DDT = sum of p,p'-DDT, o.p'-DDT, p-p'-DDE and p,p'-TDE (DDD) expressed as DDT; [c]α-Endosulfan = sum of alpha- and beta-isomers and endosulfan-sulfate expressed as endosulfan; [d]Aldrin = Aldrin and Dieldrin combined expressed as dieldrin; [e]Vinclozolin = sum of vinclozolin and all metabolites containing the 3,5-dichloraniniline moiety, expressed as vinclozolin; [f]Heptachlor = sum of heptachlor and heptachlor epoxide expressed as heptachlor; [g]Cyfluthrin = cyfluthrin including other mixtures of constituent isomers (sum of isomers); [h]Carbofuran = sum of carbofuran (including any carbofuran generated from carbosulfan, benfuracarb or furathiocarb) and 3-OH carbofuran expressed as carbofuran. [i]Carbofuran = sum of carbofuran and 3-hydroxy-carbofuran expressed as carbofuran; [j]Deltamethrin = cis-deltamethrin; [l]Disulfoton = sum of disulfoton, disulfotonsulfoxide and disulfotonsulfone expressed as disulfoton; [m]Fenamiphos = sum of fenamiphos and its sulphoxide and sulphone expressed as fenamiphos.

According to recent publications in the scientific literature, the presence of pesticides in honey has been investigated in several countries, among them, China, Serbia, Egypt, Thailand, Iran, Greece, Colombia, Malaysia, Brazil, Italy, Poland, Spain, Bosnia and Herzegovina, France, Argentina, United States, Turkey and India. Table 2 summarizes the levels of pesticides found in commercial honeys produced in these countries. High occurrence of coumaphos pesticides was observed by Wiest et al. (2011) – 77% and Balayiannis and Balayiannis (2008) – 74%; of carbendazim by Wiest et al. (2011) – 64%; and of clothianidin by Kujawski and Namiesnik (2011) – 65%. The metabolite of amitraz, N-2,4-dimethylphenyl-N-methylformamidine, was detected in 127 honey samples in concentrations up to 20 µg/kg, lower than the MRL. High percentages of honey samples from Hungary, China, United States, Argentina and Japan tested positive for this compound (92%, 81%, 60%, 58% and 32%, respectively). These results indicate that this compound has been used frequently (NAKAJIMA et al., 2015). Some other studies found high prevalence of chlorophenols, triazole, chlorpyrifos ethyl, malathion and pyrethroids in honey; however the number of samples analyzed was small (≤ 6) and was not statistically representative (MUKHERJEE, 2009; LI et al., 2013; FARAJZADEH et al., 2014; ORSO et al., 2014; FAN et al., 2015). Wang et al. (2010) and Yavuz et al. (2010) found organochlorine pesticides in 100% of the samples analyzed, while Kujawski et al. (2012) found in 79%. Hexachlorobenzene (68%) and dicofol (38.9%) were the organochlorine pesticides most frequently detected in the studies of Malhat et al. (2015) and Eissa et al. (2014), respectively. This result is worrisome because organochlorines are persistent and bioaccumulate in the environment, reason why they have been restricted or banned for agriculture use since 1978 in the USA and Europe (RIAL-OTERO et al., 2007). According to Eissa et al. (2014) dicofol is used to control *Varroa destructor*. Other acaricides used by beekeepers against *Varroa destructor* were also detected in this study (i.e., bromopropylate, tetradifon, malathion). Of the samples evaluated, 81.8% of the detected pesticides exceeded the European Union MRLs.

Table 2. Occurrence and levels of pesticides in commercial honey from different countries

Classes of pesticides analyzed	Samples		Range (µg/kg)	Country (Reference)
	Analyzed	Positive (%)		
Acylamino acid, benzofuran, anilinopyrimidines, carbamates, aryloxyphenoxypropionates, benzimidazoles, carbanilate, carboxamides, chloroacetamides, cyanoimidazole, imidazoles, diacylhydrazines, oxadiazine, dicarboximides, dinitroaniline, hydroxyanilide, morpholines, neonicotinoids, pyrethroids, organophosphorous, pyridines, phenylamides, phenylpyrazoles, phenylureas, phosphorothiolate, pyrazoles, pyridazinone, pyrimidines, ureas strobilurins, sulphite ester, triazines, tetrazine, tetronic acid, triazoles and others pesticides unclassified	66	1.5	nd - 0.029	Brazil (TETTE et al., 2016b)
Amidines, diphenyl oxazoline, pyrazole, phenylpyrazole, carboxamide, sulphite ester, pyridazinone, tetronic acid	250	50.8**	nd - 20.0	Different countries (NAKAJIMA et al., 2015)
Organophosphorous, sulphite ester, benzilate, bridged diphenyl, pyrethroid	5	20	nd - < LOQ	Iran (ZALI et al., 2015)
Chlorophenols	4	75	nd - 29.64	China (FAN et al., 2015)
Neonicotinoids	104	4.8**	nd - 29.12	Serbia (JOVANOV et al., 2015)
Organochlorines, pyrethroids	100	68**	nd - 30.6	Egypt (MALHAT et al.,2015)
Organophosphorous	39	10**	nd - 5.2	Egypt (NAGGAR et al., 2015)
Neonicotinoids	3	0	nd	Thailand (VICHAPONG et al., 2015)
Neonicotinoids, carbamates, amidine, triazines, strobilurins, carboxamide, benzimidazole, triazolone, organophosphorous, triazoles, anilinopyrimidines, benzoylurea, morpholine, amine, pyrethroids, oxadiazine, urea, imidazole, dicarboximide, phenylamide, diacylhydrazine, chloroacetamide, dinitroaniline, cyclic aromatic, tetronic acid, diacylhydrazine and others unclassified	76	97	<MRL	Different countries (COTTON et al., 2014)

Table 2. Occurrence and levels of pesticides in commercial honey from different countries (continuation…)

Classes of pesticides analyzed	Samples		Range (µg/kg)	Country (Reference)
	Analyzed	Positive (%)		
Organochlorines, organophosphorus, carbamates, pyrethroids, benzilate, pyrimidinol, chloronitrile, triazine phosphorothiolates, dicarboximides, dinitroaniline	90	55.6	10 - 1,988	Egypt (EISSA et al., 2014)
Triazoles	4	50	8.0 - 19	Iran (FARAJZADEH et al., 2014)
Neonicotinoids, dinitroanilines, triazoles, organophosphorus, carbamates, dicarboximides	13	7.7	nd - 1.62	Greece (KASIOTIS et al., 2014)
Organophosphorus, chloroacetamide, strobilurin, phthalimide, pyrethroids, cyanoacetamide oxime, triazoles, organochlorines, sulphamide, morpholine, phenylamides, chlorinated hydrocarbon, dicarboximide, phosphorothiolate, anilinopyrimidine, sulphite ester, bridged diphenyl, benzimidazole	61	52.4	nd - 54	Colombia (LOPEZ et al., 2014)
Organophosphorus, carbamates	14	0	nd	Malaysia (MONIRUZZAMAN et al., 2014)
Organophosphorus, pyrethroids, organochlorines, strobins, triazoles, chloronitrile, pyrazole, dinitroanilines	4	100	nd - 90	Brazil (ORSO et al., 2014)
Organochlorines, chlorinated hydrocarbon, organophosphorus, triazole, phthalimide, quinoline, pyrimidinol, phenylpyridinamine, strobilurin, dicarboximide, carboxamide, anthranilic diamide, tetronic acid	72	28	nd - 18.3	Italy (PANSERI et al., 2014)
Chloroacetamide, pyrazole, organophosphorus, pyrethroid, triazolone, neonicotinoid, oxadiazine, carbamate, strobilurin, phosphorothiolate, pyridazinone, aryloxyphenoxypropionate, and others unclassified	45	29	nd - 25.7	Poland (BARGANSKA et al., 2013)
Neonicotinoids	15	0	nd	Serbia (JOVANOV et al., 2013)
Pyrethroids	3	100	nd - 3.4 (µg/L)	China (LI et al., 2013)
Organochlorines, organophosphorus, carbamates, pyrethroids, dicarboximide, bridged diphenyl and others unclassified	20	nd	<LOQ	Brazil (SALAMI et al., 2013)

Table 2. Occurrence and levels of pesticides in commercial honey from different countries (continuation…)

Classes of pesticides analyzed	Samples		Range (µg/kg)	Country (Reference)
	Analyzed	Positive (%)		
Organophosphorus, organochlorines, carbamates, pyrethroids, biopesticides, ureas, neocotinoids, triazines, triazoles	26	15	nd - 5.1	Spain (GOMEZ-PEREZ et al., 2012)
Organochlorines	19	79**	nd - 13.91	Poland (KUJAWSKI et al., 2012)
Alkylchlorophenoxy, phenylurea, phenylpyrazole	2	0	nd	Brazil (SAMPAIO et al., 2012)
Amidine, benzimidazole, organophosohorus, phenylpyrazole, pyrethroid, neonicotinoid, triazole	3	nd	nd	Brazil (TOMASINI et al., 2012)
Organochlorines	nf	0	nd	Greece (ZACHARIS et al., 2012)
Organophosphorus, carbamates	25	24	nd – 296.3	Spain (BLASCO et al., 2011)
Neonicotinoid, triazolone, pyridine, phenylamide, organophosphorus, imidazole, triazole, carbamate, sulfonylurea and other unclassified	40	65	nd - 4.98	Poland (KUJAWSKI & NAMIESNIK, 2011)
Organochlorines, chlorinated hydrocarbon, organophosphorus, triazines, benzonitrile	46	nd	nd	Bosnia and Herzegovina (MUJIC et al., 2011)
Organophosphorous, organochlorines, chlorinated hydrocarbon, phthalimide, dicarboximide, benzilate, pyrimidine	17	41	nd - 13	Greece (TSIROPOULOS & AMVRAZI, 2011)
Organochlorines, amide, organophosphorus, pyrethroids, carbamates, triazoles, nicotinoids, pyrimidines, dicarboximide, imidazole, avermectins, formamidine, tetrazine, thiazolidine, synergist, insect growth regulator	142	77	nd - 116	France (WIEST et al., 2011)
Pyrethroids	3	0	nd	China (ZHANG et al., 2011)
Neonicotinoids, phenylpyrazoles	91	0	nd	Spain (GARCIA-CHAO et al., 2010)
Organophosphorus	5	40	nd - 2.3	Argentina (FONTANA et al., 2010)
Organophosphorus, pyrethroids	11	18	nd - 0.22	Brazil (PINHO et al., 2010)
Organochlorines	38	100	nd - 8.70	Different countries (WANG et al., 2010)
Organochlorines	109	100	nd - 5,024.5	Turkey (YAVUZ et al., 2010)

Table 2. Occurrence and levels of pesticides in commercial honey from different countries (continuation…)

Classes of pesticides analyzed	Samples		Range (µg/kg)	Country (Reference)
	Analyzed	Positive (%)		
Pyrethroids, organochlorines, organophosphorus	6	83	nd - 410	India (MUKHERJEE et al., 2009)
Organophosphorus	50	92	nd - 4.8	Greece (BALAYIANNIS & BALAYIANNIS, 2008)
Organochlorine, cyclodiene, pyrethroids, organophosphorus	51	35	nd - 11.08	India (CHOUDHARY et al., 2008)

nf = not found; nd = not detected; LOQ = quantification limit; MRL = maximum residue level.

* Pesticides were classified according to Pesticide Properties Database (AERU, 2015), except when this information was in the cited study.

** This value corresponds to the minimum percentage of contaminated samples. It was not possible to define the exact percentage with the data found in the cited study.

3. Methods for analysis of pesticides in honey

Several methods have been used for the analysis of pesticides in honey. The methods used were specific for a pesticide, for a class of pesticides or for several pesticides (multiclass method). However, it should be noted that the quality of the results is closely linked to an appropriate selection of the analytical method (GARCIA-CHAO et al., 2010; VICHAPONG et al., 2015). A multiresidue method suitable for detecting and quantifying the pesticides of interest in a relatively short period of time, comprising minimum steps of extraction and purification, is essential for an efficient monitoring program (ALBERO et al., 2005; RISSATO et al., 2006). Irrespective of the type of method, at least three steps are required, among them extraction, separation and detection. Each one of them will be described.

3.1. Sample preparation

Sample preparation is an important step within the entire analytical process (PRESTES et al., 2009). Generally, it is the most time-consuming and labor-intensive part of the analysis. Furthermore, it is the step most prone to

errors, which can compromise the results (KUJAWSKI & NAMIESNIK, 2011). The main objectives of sample preparation are to promote the extraction and enrichment of the analytes and remove as much as possible the interferences. According to Hercegová et al. (HERCEGOVA et al., 2007), a sample preparation method for analysis of pesticide residues should have the following properties: include the highest possible number of pesticides (multiresidue assay), have recoveries as close as possible to 100%, remove potential interfering compounds in the sample to improve selectivity, allow increasing concentration of the analytes, have appropriate precision and ruggedness, be quick, easy, safe and of low cost.

Sample preparation and enrichment with the target compounds are very important because the contaminants are present in honey at very low concentrations (BARGANSKA et al., 2013). Once honey is complex, a sample preparation step is necessary. Usually, dilution of heated honey samples with different solvents or with a mixture of solvents is the first step undertaken. Moreover, clean-up procedures have also been performed after extraction to eliminate interferences prior to analysis (GARCIA-CHAO et al., 2010). Thus, the main criterion is to find one method that gives acceptable recoveries for all analytes with only one protocol (WIEST et al., 2011).

The most common interferences present in bee extracts are carbohydrates, pigments and lipids (RISSATO et al., 2006). However, more than 300 compounds may be present in honey. This constitutes a challenge for analysts who wish to determine residual contamination levels (KUJAWSKI & NAMIESNIK, 2008). Sample preparation will depend on the type of separation and quantification method. The methods used in the extraction of pesticides from honey described in the literature from 2008 to 2015 by using gas chromatography and liquid chromatography are summarized in Tables 3 and 4, respectively.

Table 3. Sample preparation for pesticides analysis in honey employing gas chromatography

Classes of pesticides analyzed	Separation/ Detection	Extraction method	Solvent	Clean up	Sorbent	Recovery (%)	Reference
Organophosphorous, sulphite ester, benzilate, bridged diphenyl, pyrethroid (7 pesticides)	GC-MS	SPME	Sodium chloride solution	SPME	Polystyrene nanofibers	81 - 125	ZALI et al., 2015
Organochlorines, pyrethroids (18 pesticides)	GC-μECD	QuEChERS	Acetonitrile acidified with acetic acid	dSPE	Magnesium sulfate and PSA	85 - 115	MALHAT et al., 2015
Organochlorines, organophosphorus, carbamates, pyrethroids, benzilate, pyrimidinol, chloronitrile, triazine phosphorothiolates, dicarboximides, dinitroaniline (46 pesticides)	GC-ECD GC-NPD	QuEChERS	Acetonitrile acidified with acetic acid	dSPE	Magnesium sulfate and PSA	84 - 120	EISSA et al., 2014
Triazoles (5 pesticides)	GC-NPD	ET-DLLME	Dimethylformamide and 1,2-dibromoethane	-	-	97 - 100	FARAJZADEH et al., 2014
Organophosphorus, chloroacetamide, strobilurin, phthalimide, pyrethroids, cyanoacetamide oxime, triazoles, organochlorines, sulphamide, morpholine, phenylamides, chlorinated hydrocarbon, dicarboximide, phosphorothiolate, anilinopyrimidine, sulphite ester, bridged diphenyl, benzimidazole (53 pesticides)	GC-NPD and μECD	LLE	Ethyl acetate	SPE	Magnesium silicate and silica	34 - 119	LOPEZ et al., 2014
Organophosphorus, organochlorines, pyrethroids, strobins, triazoles, chloronitrile, dinitroanilines, pyrazole (24 pesticides)	GC-ECD	QuEChERS	Acetonitrile	dSPE	Magnesium sulfate and PSA	71 - 119	ORSO et al., 2014
Organochlorines, chlorinated hydrocarbon, organophosphorus, triazole, phthalimide, pyrimidinol, quinoline, phenylpyridinamine, strobilurin, dicarboximide, carboxamide, anthranilic diamide, tetronic acid (28 pesticides)	GC-MS/MS	LLE	Ethyl acetate	SPE	Magnesium silicate/ Na_2SO_4	75 - 102	PANSERI et al., 2014

Table 3. Sample preparation for pesticides analysis in honey employing gas chromatography (continuation…)

Classes of pesticides analyzed	Separation/ Detection	Extraction method	Solvent	Clean up	Sorbent	Recovery (%)	Reference
Organochlorines (8 pesticides)	GC-ECD	MSPE	Ethyl acetate	MSPE	Magnetic cobalt ferrite filled carbon nanotubes (MFCNTs)	83 - 128	DU et al., 2013
Organochlorines, organophosphorus, carbamates, pyrethroids, dicarboximide, bridged diphenyl and others unclassified (22 pesticides)	GC-MS	LLE	Ethyl acetate and hexane	MEPS	C8 and M1	82 - 114	SALAMI et al., 2013
Organotins (7 pesticides)	GC-AED GC-MS	HS-SPME	NaCl, acetate buffer solution and sodium tetraethylborate	SPME	Polydimethyl siloxane (PDMS)	77 - 101	CAMPILLO et al., 2012
Organonitrogens (9 pesticides)	GC-NPD GC-MS	Purge and trap	-	-	Polydivinyl-benzene Silica monolithic	85 - 100	CHIENTHAVORN et al., 2012
Organochlorines (11 pesticides)	GC-MS	DLLME	Acetone and chloroform	-	-	35 - 83	KUJAWSKI et al., 2012
Organochlorines (15 pesticides)	GC-ECD GC-IT-MS	DLLME	Acetonitrile and chloroform	-	-	75 - 119	ZACHARIS et al., 2012
Pyrethroid (1 pesticide)	GC-MS	SPE	Water/acetone	-	C18	87	BONZINI et al., 2011
Organochlorines, chlorinated hydrocarbon, organophosphorus, triazines, benzonitrile (18 pesticides)	GC-MS	LLE	Acetonitrile	SPE	C18	nf	MUJIC et al., 2011
Organophosphorous, organochlorines, chlorinated hydrocarbon, phthalimide, dicarboximide, benzilate, pyrimidine (14 pesticides)	GC-ECD GC-MS	SDME	Toluene Xylene	-	-	71 – 120	TSIROPOULOS & AMVRAZI, 2011

Table 3. Sample preparation for pesticides analysis in honey employing gas chromatography (continuation…)

Classes of pesticides analyzed	Separation/ Detection	Extraction method	Solvent	Clean up	Sorbent	Recovery (%)	Reference
Organochlorines, amide, organophosphorus, pyrethroids, triazoles, pyrimidines, dicarboximide, synergist, insect growth regulator (80 pesticides)	GC-TOF	QuEChERS	Acetonitrile	dSPE	magnesium sulfate and PSA	23 - 136	WIEST et al., 2011
Organophosphorus, organochlorines, pyrethroids, n-methyl-carbamates, bromopropylate (53 pesticides)	GC-FPD GC-MS LC/DD/FI	SPE	Water/Acetone dichloromethane	-	Diatomaceous earth	70 - 103	AMENDOLA et al., 2011
Organophosphorus (4 pesticides)	GC-MS	CME-UABE	Triton X-114	-	-	90 - 107	FONTANA et al., 2010
Organophosphorus, pyrethroids (4 pesticides)	GC-ECD GC-MS	LLE-LTP	Acetonitrile/ethyl acetate	SPE	Magnesium silicate	84 - 100	PINHO et al., 2010
Organochlorines (11 pesticides)	GC–IT/MS	ASE	Acetone/ methylene chloride	SPE	Neutral alumina (3 cm, 3% deactivated), neutral silica gel (4 cm, 3% deactivated), 50% sulphuric acid silica (2 cm), and anhydrous sodium sulfate (2 cm).	52 - 95	WANG et al., 2010
Organochlorines (24 pesticides)	GC-ECD	LLE	Light petroleum	SPE	Magnesium silicate	77 - 105	YAVUZ et al., 2010
Pyrethroids, organochlorines, organophosphorus (8 pesticides)	GC-ECD	LLE	Ethyl acetate	SPE	Magnesium silicate	60 - 90.6	MUKHERJEE, 2009
Organophosphorus (5 pesticides)	GC-FPD	SBSE	Acetonitrile	-	PDMS/poly (vinylalcohol) (PDMS/PVA) film	12 - 124	YU & HU, 2009

Table 3. Sample preparation for pesticides analysis in honey employing gas chromatography (continuation…)

Classes of pesticides analyzed	Separation/ Detection	Extraction method	Solvent	Clean up	Sorbent	Recovery (%)	Reference
Organophosphorus (10 pesticides)	GC-NPD	LLE	Methylene chloride	-	-	73 - 104	BALAYIANNIS & BALAYIANNIS, 2008
Organochlorines, cyclodiene, pyrethroids, organophosphorus (20 pesticides)	GC-ECD	LLE	Water/ methanol	SPE	Magnesium silicate and silical gel mixture	76 - 91	CHOUDHARY et al., 2008

Legend: "-" = not applicable; ASE = accelerated solvent extraction; AED = atomic electron detector; CME-UABE = coacervative microextraction ultrasound-assisted back-extraction; DD = double derivatization; DLLME = dispersive liquid-liquid microextraction; dSPE = dispersive solid phase extraction; ECD = electron capture detector; ET = elevated-temperature; FI = spectrofluorimetric detector; FPD = flame photometric detector; GC = gas chromatography; HS-SPME = headspace solid-phase microextraction; IT = ion trap; IT/MS = ion trap mass spectrometry; LLE = liquid-liquid extraction; LTP = low temperature Purification; MEPS = microextraction by packed sorbent; MS = mass spectrometry; MS/MS = tandem mass spectrometry; MSPE = magnetic solid-phase extraction; nf = not found; NPD = nitrogen phosphorus detector; PSA = primary secondary amine; QuEChERS = quick, easy, cheap, effective, rugged and safe; SBSE = stir bar sorptive extraction; SDME = single-drop microextraction; SPE = solid phase extraction; SPME = solid phase microextraction; ToF = time-of-flight; LC/DD/FI = liquid chromatography-double derivatization coupled with spectrofluorimetric detector.

Table 4. Sample preparation for pesticides analysis in honey employing liquid chromatography

Classes of pesticides analyzed	Separation/ Detection	Extraction method	Solvent	Clean up	Sorbent	Recovery (%)	Reference
Acylamino acid, benzofuran, anilinopyrimidines, carbamates, aryloxyphenoxypropionates, benzimidazoles, carbanilate, carboxamides, chloroacetamides, cyanoimidazole, diacylhydrazines, dicarboximides, dinitroaniline, hydroxyanilide, imidazoles, morpholines, neonicotinoids, organophosphorous, oxadiazine, phenylamides, phenylpyrazoles, phenylureas, phosphorothiolate, pyrazoles, pyrethroids, pyridines, pyridazinone, pyrimidines, ureas strobilurins, sulphite ester, triazines, tetrazine, tetronic acid, triazoles and others pesticides unclassified (116 pesticides)	LC-MS/MS	QuEChERS	Acetonitrile, ethyl acetate	dSPE	PSA and magnesium silicate	81.6 - 108.9	TETTE et al., 2016b
Amidines, diphenyl oxazoline, pyrazole, phenylpyrazole, carboxamide, sulphite ester, pyridazinone, tetronic acid (9 pesticides)	LC-MS/MS	LLE	Ethyl acetate	dSPE	Quaternary Ammonium Group Introduction Strong Anion Exchange Polymer	77 – 116	NAKAJIMA et al., 2015
Chlorophenols (6 pesticides)	HPLC-UV	IL-DLLME	Trifluoromethyl sulfonyl)imide anion $[C_4MIM][NTf_2]$ Trifluoromethyl sulfonyl tetrafluoroborate anion $[C_4MIM][BF_4]$	-	-	91 – 114	FAN et al., 2015

Table 4. Sample preparation for pesticides analysis in honey employing liquid chromatography (continuation…)

Classes of pesticides analyzed	Separation/ Detection	Extraction method	Solvent	Clean up	Sorbent	Recovery (%)	Reference
Neonicotinoids (7 pesticides)	HPLC-UV	DLLME	Acetonitrile and dichloromethane	-	-	73 – 118	JOVANOV et al., 2015
Organophosphorous (14 pesticides)	LC-MS/MS	QuEChERS	Acetonitrile Deionized water Glacial acid acetic	SPE dSPE	SPE: C18 dSPE: magnesium sulfate and PSA	86 – 106	NAGGAR et al., 2015
Neonicotinoids (7 pesticides)	HPLC-UV	IS-DLLME	Sodium sulfate 10% (w/v) and 1-octanol	-	-	96 – 107	VICHAPONG et al., 2015
Neonicotinoids, carbamates, amidine, triazines, strobilurins, carboxamide, benzimidazole, triazolone, organophosphorous, triazoles, anilinopyrimidines, benzoylurea, morpholine, amine, pyrethroids, oxadiazine, urea, imidazole, dicarboximide, phenylamide, diacylhydrazine, chloroacetamide, dinitroaniline, cyclic aromatic, tetronic acid, diacylhydrazine and others unclassified (55 pesticides)	LC-MS/MS	QuEChERS	Acetonitrile	-	-	nf	COTTON et al., 2014
Neonicotinoids, dinitroanilines, triazoles, organophosphorous, carbamates, dicarboximides (115 pesticides)	LC-MS/MS	QuEChERS	Acetonitrile (with and without triethylamine)	dSPE	Magnesium sulfate, sodium acetate and PSA	59 - 117	KASIOTIS et al., 2014

Table 4. Sample preparation for pesticides analysis in honey employing liquid chromatography (continuation…)

Classes of pesticides analyzed	Separation/ Detection	Extraction method	Solvent	Clean up	Sorbent	Recovery (%)	Reference
Anilinopyrimidine, aryloxyphenoxypropionate, anilinopyrimidine, pyrazole, organophosphorus, strobilurin, pyridazinone, cyanoacetamide oxime, benzoylurea, morpholine, quinazoline, triazole, pyretroids, pyrazole, oxathiin, carbamate, chloroacetamide, triazine, thiocarbamate and others unclassified (30 pesticides)	LC-ESI-MS/MS	SLE QuEChERS	SLE: ethyl acetate QuEChERS: acetonitrile	SLE: SPE QuEChERS: -	SLE: diatomaceous earth QuEChERS: -	SLE: 36 - 96 QuEChERS: 36 - 86	KUJAWSKI et al., 2014
Organophosphorus, carbamates (10 pesticides)	HPLC-UV	LLE	Dichloromethane	SPE	Magnesium silicate	70 - 84	MONIRUZZAMAN et al., 2014
Chloroacetamide, pyrazole, organophosphorus, pyrethroid, triazolone, neonicotinoid, oxadiazine, carbamate, strobilurin, phosphorothiolate, pyridazinone, aryloxyphenoxypropionate, and others unclassified (30 pesticides)	LC-ESI-MS/MS	QuEChERS	Acetonitrile	dSPE	magnesium sulfate and PSA	80 - 109	BARGANSKA et al., 2013
Neonicotinoids (6 pesticides)	UHPLC/MS-MS	QuEChERS	Acetonitrile	-	-	75 - 114	GALEANO et al., 2013
Neonicotinoids (7 pesticides)	LC-MS/MS	DLLME	Acetonitrile and dichloromethane	-	-	74 - 113	JOVANOV et al., 2013

Table 4. Sample preparation for pesticides analysis in honey employing liquid chromatography (continuation…)

Classes of pesticides analyzed	Separation/ Detection	Extraction method	Solvent	Clean up	Sorbent	Recovery (%)	Reference
Pyrethroids (4 pesticides)	HPLC-UV	IL-DLLME	Ionic liquid [C_6MIM]NTf_2: 1-Hexyl-3-methylimidazolium chloride ([C_6MIM]Cl) trifluoromethane sulfonimide) ($LiNTf_2$)	D-µ-SPE	Non-modified magnetic nanoparticles (MNPs)	86 - 98	LI et al., 2013
Organophosphorus, organochlorines, carbamates, pyrethroids, biopesticides, ureas, neocotinoids, triazines, triazoles (350 pesticides)	UHPLC-Orbitrap-MS	LLE	Acetonitrile	-	-	66 - 102	GÓMEZ-PÉREZ et al., 2012
Alkylchlorophenoxy, phenylurea, phenylpyrazole (3 pesticides)	HPLC-UV LC-ESI-MS/MS	QuEChERS	Acetonitrile	dSPE	Magnesium sulfate and PSA	HPLC-UV: 63 - 114 LC-ESI-MS/MS 90 - 120	SAMPAIO et al., 2012
Amidine, benzimidazole, organophosohorus, phenylpyrazole, pyrethroid, neonicotinoid, triazole (8 pesticides)	LC-APCI-MS/MS	QuEChERS	Acetonitrile	dSPE	PSA	70 - 112	TOMASINI et al., 2012
Neonicotinoid, triazolone, pyridine, phenylamide, organophosphorus, imidazole, triazole, carbamate, sulfonylurea and other unclassified (13 pesticides)	LC-ESI-MS/MS	LLE	Acetonitrile	SPE	Diatomaceous earth	63 - 117	KUJAWSKI & NAMIESNIK, 2011

Table 4. Sample preparation for pesticides analysis in honey employing liquid chromatography (continuation…)

Classes of pesticides analyzed	Separation/ Detection	Extraction method	Solvent	Clean up	Sorbent	Recovery (%)	Reference
Amide, organophosphorus, carbamates, triazoles, nicotinoids, dicarboximide, imidazole, avermectins, formamidine, tetrazine, thiazolidine, synergist, insect growth regulator (80 pesticides)	LC-MS/MS	QuEChERS	Acetonitrile	dSPE	Magnesium sulfate and PSA	60 - 120	WIEST et al., 2011
Pyrethroids (4 pesticides)	HPLC-UV	UA- IL-DLLME	Methanol and 1-octyl-3-methylimidazolium hexafluoro phosphate [C8MIM][PF6]	-	-	101 - 103	ZHANG et al., 2011
Neonicotinoids, phenylpyrazoles (7 pesticides)	LC-MS/MS	SPE	Methanol/Water	-	Magnesium silicate	89 - 102	GARCIA-CHAO et al., 2010
Neonicotinoids (12 pesticides)	UPLC-MS/MS	QuEChERS	Acetonitrile with 2% triethylamine	SPE	C18	70 - 120	KAMEL, 2010
Organophosphorus, benzimidazole, amidine (4 pesticides)	LC-MS/MS	SPE	Hydrochloric acid	-	Poly(divinylbenzene-N-vinylpyrrolidone)	40 - 102	DEBAYLE et al., 2008
Carbamates (3 pesticides)	LC-ESI-MS	LLE	Ethyl acetate	-	-	94.8 - 109.2	ZHU et al., 2008

Legend: "-" = not applicable; APCI = atmospheric pressure chemical ionization; UV = ultraviolet detector; DLLME = dispersive liquid-liquid microextraction; dSPE = dispersive solid phase extraction; ESI = electrospray ionization; HPLC = high-performance liquid chromatography; IL = ionic liquid; LLE = liquid liquid extraction; MS = mass spectrometry; MS/MS = tandem mass spectrometry; nf = not found; PSA = primary secondary amine; QuEChERS = quick, easy, cheap, effective, rugged and safe; SLE = solid supported liquid-liquid extraction; SPE = solid phase extraction; UA = ultrasound-assisted; UHPLC = ultra high performance liquid chromatography; UA = ultrasound-assisted ionic liquid dispersive liquid liquid microextraction.

3.1.1. Liquid liquid extraction

Liquid-liquid extraction (LLE) is the most common extraction and purification technique used in the determination of pesticides in honey (BLASCO et al., 2004; PIRARD et al., 2007). LLE usually employs large sample sizes and toxic organic solvents. It is also characterized by the use of multiple sample handling steps, which makes it susceptible to error and contamination (ANTHEMIDIS & IOANNOU, 2009; PENA-PEREIRA et al., 2009). Furthermore, it usually enables the extraction of analytes belonging to only one chemical class (KUJAWSKI et al., 2014).

Despite the disadvantages described above, LLE continues to be used in the analysis of pesticides in honey. The most widely used organic solvents are ethyl acetate, acetonitrile and methanol (KUJAWSKI & NAMIESNIK, 2011; SALAMI et al., 2013; LOPEZ et al., 2014; PANSERI et al., 2014) but other solvents have also been used, as is the case of the study described by Yavuz et al. (YAVUZ et al., 2010) that extracted organochlorine pesticides from honey using light petroleum and observed recoveries in the range of 77.3 to 105.2%. According to Debayle et al. (2008), an adaptation of the sample preparation method depending on the physicochemical characteristic of each pesticide is needed. In this method, hydrochloric acid was used during extraction because the antibacterial sulfonamides were extracted together with the pesticides.

The use of different ratios of solvents and aqueous solutions can also affect extraction performance. Interferences such as carbohydrates or pigments can be co-extracted and affect the recovery of the target compounds when high solvent volume is used. This behavior was observed by Goméz-Pérez et al. (2012) during the extraction of more than 350 compounds, including pesticides and antibiotics, from honey. Acetonitrile allowed the extraction of more compounds than acetone and the ratio which enabled the best results was 2.5 mL of water + 7.5 mL of acetonitrile. The method validated by these authors had no additional clean-up.

LLE is frequently used together with a clean-up in a column of solid phase extraction (SPE) (CHOUDHARY & SHARMA, 2008; DEBAYLE et al., 2008). The most commonly used sorbent for honey clean up after LLE has been magnesium silicate, although others have been also used such as

octadecylsilane, octylsilane, silica gel, polydimethylsiloxane, polydivinylbenzene, silica monolithic, diatomaceous earth and primary secondary amine (PSA) (CHOUDHARY & SHARMA, 2008; YAVUZ et al., 2010; KUJAWSKI & NAMIESNIK, 2011; LOPEZ et al., 2014; PANSERI et al., 2014). Magnesium silicate has been extensively used because it has proved to be very efficient for the clean-up of food samples (MONIRUZZAMAN et al., 2014).

Although liquid-liquid extraction is considered as a conventional technique, innovations to increase its efficiency in honey have been developed. Kujawski and Namiesnik (2011) conducted a study to determine 13 multi-class pesticides residues in honey using LLE on a diatomaceous earth support. The procedure involved the introduction of a mixture of water and acetonitrile with a certain amount of sodium chloride to the column and the extraction of the analytes from a thin layer of the liquid, adsorbed on a diatomaceous earth support, with an organic solvent (e.g. dichloromethane or ethylene acetate). The eluate was evaporated to dryness and reconstituted with methanol/water (70:30, v/v). This method was applied to 40 samples and recoveries were in the range of 63 - 117%.

Another innovation in this direction was the use of low temperatures. Liquid-liquid extraction and low temperature (LLE-LTP) was used by Goulart et al. (2008) in the analysis of deltamethrin and cypermethrin in milk. The LLE-LTP procedure consisted of adding acetonitrile to the sample in a ratio of 2:1 (v/v). After addition of acetonitrile, a single liquid phase containing water and acetonitrile was formed. The mixture was cooled to -20 °C and the solid material was trapped in the frozen water phase, whereas acetonitrile, which freezes at -46 °C, remained liquid at -20 °C and could be easily removed. This method showed high recovery for the studied pyrethroids (> 80 %). Currently, several studies are based on the use of this technique in the extraction and clean-up of different compounds of various matrices, including honey. Pinho et al. (2010) proposed the use of LLE-LTP for the extraction of chlorpyrifos, k-cyhalothrin, cypermethrin and deltamethrin in honey. The authors optimized the amount of sample, homogenization techniques, addition of magnesium silicate (a second clean up stage) and the composition of the extracting solution. Increased amounts of honey led to higher recoveries; however when 4 g sample was used, the biphasic system (4 g of sample and 8 mL of extraction solution) was

not solidified after the freezing step (-20 °C). Four compositions of the extracting solutions were tested: hexane/ethyl acetate (4:4, v/v); acetonitrile/ethyl acetate (4:4); acetonitrile; and acetonitrile/ethyl acetate (6.5:1.5, v/v). Best recoveries (>70%) were observed with the last one. Since honey produced an emulsion in aqueous solution during the homogenization step, other homogenization techniques were tested: manual shaking, ultrasonic bath, vortexing and shaking table. Vortexing for 30 seconds provided the best results. The use of a clean-up step was important to eliminate interferences. The addition of salt (NaCl) increased the recovery of chlorpyrifos, but not of the other pesticides. The optimized method was applied to 11 honey samples from eight regions of the state of Minas Gerais (Brazil). Chlorpyrifos residue was found in two samples. However, the concentrations obtained were below the MRL established for pesticides in foods.

3.1.2. Solid phase extraction

Solid phase extraction (SPE) is based on the retention of selected analytes on sorbents followed by their elution with appropriate solvents (PIRARD et al., 2007). It combines extraction and clean-up procedures in a single step, providing clean extracts, which can be directly analyzed by GC or LC (AMENDOLA et al., 2011). Simplicity, robustness, relative speed and low solvent consumption are characteristics that make this technique an attractive alternative in the analysis of complex matrices (PIRARD et al., 2007).

The extraction of pesticides from honey has been performed by means of poly(divinylbenzene-co-N-vinylpyrrolidone) copolymer (DEBAYLE et al., 2008), diatomaceous earth (AMENDOLA et al., 2011), magnesium silicate (GARCIA-CHAO et al., 2010) and C18 cartridges (BONZINI et al., 2011). Amendola et al. (2011) used a SPE based method for the analysis of 53 pesticides of different classes in honey including those used to control *Varroa* and others, arising from crop protection. The developed method was fast and used low amount of solvent. In this method, honey was dissolved in water-acetone, loaded into a diatomaceous earth sorbent and distributed along the column by gravity. Most of the co-extractive compounds were retained on the adsorbent material, while

the pesticides were eluted by dichloromethane and directly analyzed by gas and liquid chromatography.

SPE also proved to be efficient for the analysis of raw samples taken directly from the apiary. To separate fractions from the raw honey, García-Chao et al. (2010) submitted the samples to centrifugation. The honey portion went to the bottom whereas, on the top of the tube, there was a mixture of waxes, pollen and larvae. The top layer was discarded and the rest, a mixture of honey with small portions of residual pollen and wax, was used for extraction. Fipronil and some of its metabolites (fipronil sulfone, fipronil sulfide, fipronil desulfinyl and fipronil carboxamide), thiamethoxam and imidacloprid were extracted from honey after optimization using a Doehlert experimental design. The optimized method consisted of 1 g of honey mixed with 3 mL of the optimized mixture of methanol:water (10:90, v/v). The extract was passed through a magnesium silicate cartridge and the target compounds were eluted with methanol and analyzed by LC-MS/MS.

3.1.3. QuEChERS

QuEChERS, an acronym for Quick, Easy, Cheap, Effective, Rugged and Safe, was developed by Anastassiades et al. in 2003. During its development, great emphasis has been given to obtain a dynamic procedure that could be applied in any laboratory, due to the simplification of the steps (PRESTES et al., 2009). This method has become a popular technique for sample preparation at an international level (CIESLIK et al., 2011). The QuEChERS multiresidue procedure replaces many complicated analytical steps commonly employed in traditional methods by easier ones (KOLBERG et al., 2011). The method is based on the use of magnesium sulfate ($MgSO_4$) and sodium chloride (NaCl) for the extraction/separation of the compounds of interest by salting out and dispersive solid phase extraction (dSPE) to clean the samples (PRESTES et al., 2009).

Although the first version of the method has shown excellent results for hundreds of pesticides in various matrices, further experiments showed that some pesticides' stability or recovery increased depending on the pH of the matrix (ANASTASSIADES et al., 2003; LEHOTAY et al., 2005; PAYA et al.,

2007). However, modifications of the pH are not frequent for all matrices. For honey, for example, changes on the type of solvent used in the extraction step (acetonitrile, ethyl acetate, methanol and acetone) and in the amount of sample (0.5 to 10 g) are more common (SAMPAIO et al., 2012; KUJAWSKI et al., 2014).

QuEChERS has been the most commonly used method for the analysis of pesticides in honey. Modifications in the original QuEChERS have been carried out depending on the pesticides analyzed and the characteristics of the sample (KAMEL, 2010; GALEANO et al., 2013). In this context, experimental protocol with the addition of triethylamine in acetonitrile has been used with good results for certain pesticides. According to Kasiotis et al. (2014), triethylamine was satisfactory for clothianidin, thiamethoxam, imidacloprid and carbendazim analysis, resulting in better peak shape, symmetry, and resolution. Triethylamine provides a basic pH preventing the protonation of basic pesticides, consequently reducing peak tailing in the chromatographic analysis.

Another modification of original QuEChERS found in the literature is the substitution of the original sorbent. PSA is known to retain pesticides containing carboxylic acid groups, as is the case of the imidacloprid metabolite 6-chloronicotinic acid. According to Kamel et al. (2010), 6-chloronicotinic acid was completely unrecovered because it was bound to PSA. To solve this problem, PSA was replaced with C18 SPE cartridges, allowing the extraction of this metabolite.

Kujawski et al. (2014) compared the extraction efficiency for 30 pesticides belonging to over 15 different chemical classes, by means of two extraction approaches: QuEChERS and solid supported liquid-liquid extraction (SLE) on diatomaceous earth. The authors concluded that the modified QuEChERS had advantages over the SLE method in terms of time consumption, since the procedure did not require evaporation. Moreover, less toxic solvent with lower volume was used (acetonitrile - 10 mL instead of methylene dichloride – 15 mL). Sample preparation times were about 20 min for QuEChERS and 60 min for SLE, but in the latter, the steps consisted of mainly waiting for equilibration on the support and then for total evaporation. However, as the performances of both methods, in terms of repeatability and detectability, were similar, it is a matter of preference.

Despite the wide use of QuEChERS in the analysis of pesticides in honey, this approach has disadvantages. One limitation is that the sample should have more than 75% of water, so an initial dissolution of honey sample is required (KUJAWSKI et al., 2014), which leads to lower concentration of the sample compared to other sample preparation techniques (WIEST et al., 2011). In order to overcome this limitation, Wiest et al. (2011) opted to add a sample concentration step by evaporation which was satisfactory for extraction of organohalogens, organophosphorous, pyrethroid, and insect growth regulator in honey. According to these authors, evaporation may be necessary when the MRL is lower than the method LOD. The evaporation step was also adopted by Jovanov et al. (2015) and Cotton et al. (2014).

3.1.4. Purge and trap

The purge and trap technique using a monolithic adsorbent in a capillary was applied by Chienthavorn et al. (2012) to extract pesticides from honey. The method consisted in adding 4 g of honey and 8 g of water into a vial. The vial was closed and a silica monolith capillary was introduced through a septum. The mixture was gently stirred while heating at 100 °C, and purged with a 4 mL/min N_2 gas flow for 60 min to evaporate the volatile pesticides, which were trapped by the monolith. The monolith capillary was then put in an oven at 40 °C for 60 min to remove moisture and was subsequently submitted to chromatography. Recovery was in the range of 84.95-99.71% and LOD of the pesticides determined using GC-NPD and GC-MS ranged between 0.36-1.75 and 0.13-0.25 ng/g, respectively.

Compared to other extraction techniques coupled to gas chromatography, purge and trap with monolith sorbent allowed obtaining detection limits lower than the other reported methods for all selected pesticides. LOD was 1.3-23.5 times lower than those obtained with solvent extraction and SPE and about 1.5 orders of magnitude lower than those with SFE coupled to GC-ECD. This study showed that purge and trap is a very promising technique, providing extremely low detection limits for organonitrogen pesticides in honey (CHIENTHAVORN et al., 2012).

3.1.5. Miniaturized techniques

3.1.5.1. Dispersive liquid liquid microextraction

Dispersive liquid liquid microextraction (DLLME), a miniaturized technique developed by Rezaee and collaborators in 2006, has also been extensively used in the determination of pesticides in honey (REZAEE et al., 2006). In DLLME, extraction and dispersive solvents are simultaneously and rapidly injected into the aqueous sample using a syringe. The analyte is extracted from the sample by means of fine droplets in the extraction solvent. Afterwards, centrifugation is used to promote phase separation and allows the enrichment of the analyte (CHEN et al., 2008).

The miscibility of the dispersing solvent and the aqueous phase is the most important factor in DLLME (CHEN et al., 2008). Thus, an appropriate optimization of the parameters affecting the procedure is crucial. Zacharis et al. (2012) optimized the type and volume of the extraction and disperser solvents, the sample pH and ionic strength, the extraction time and the centrifugation speed to determine organochlorine pesticides (OCPs) in honey. Three organic solvents with different water-solubilities, namely dichloromethane (CH_2Cl_2), chloroform ($CHCl_3$) and tetrachloroethylene (C_2Cl_4) were investigated as extracting solvent. For the majority of the analytes the extraction efficiencies followed the order CH_2Cl_2 < $CHCl_3$ < C_2Cl_4. Four disperser solvents (acetonitrile, methanol, acetone and tetrahydrofuran) were investigated. Acetone and tetrahydrofuran had similar results with low extraction efficiency for most of the pesticides. Acetonitrile provided 6-14% higher extraction efficiency when compared to methanol. The volume of the disperser solvent (acetonitrile) was investigated from 250 to 1500 µL and 750 µL was selected as the optimum. After optimization and validation, the enrichment factors ranged from 36 to 114. The limits of detection (LOD) were in the range of 0.2-4.0 ng/g when gas-chromatography coupled to ion trap mass spectrometric detection was used.

An interesting approach is the use of chemometric tools for the optimization of DLLME parameters. This procedure was employed on the extraction of organochlorine pesticides in honey by Kujawski et al. (2012). Some factors affecting recovery were optimized, among them, the type and

volume of extraction and disperser solvents, the salt content and pH. The limit of quantification ranged from 0.3 to 13.2 ng/g, which are below the MRL of the European Community and the recoveries reached a maximum of 83%. The developed method allowed the determination of organochlorine pesticides in honey, with minor amounts of organic solvents, limits of quantification comparable or lower than those observed with conventional methods, and short time.

Variations of the original DLLME method have also been used in the analysis of pesticides in honey. Li et al. (2013) developed a novel microextraction technique - ionic liquid-linked dual magnetic microextraction (IL-DMME) - for the determination of pyrethroids in honey. The distinct advantage of the method is that high recoveries can be readily achieved through the combination of DLLME with dispersive microsolid-phase extraction (D-µ-SPE) by using synthetic ionic liquid and non-modified magnetic nanoparticles (MNPs). The most common modifications in DLLME are ultrasound-assisted (UA) and temperature-controlled (TC) techniques. The UA method was tested by Zhang et al. (2011) and was considered a valuable option for the determination of pesticides in honey. Since high temperatures are a driving force for better dispersion of the extraction solvent in the aqueous phase, it can help reach higher enrichment factors and extraction recoveries. This effect was observed by Farajzadeh et al. (2014) who claimed that the method has many merits such as very low LOD, excellent sensitivity, shorter extraction time, and higher repeatability and reproducibility. As a conclusion, the developed method could be used for the determination of ultra-trace triazole pesticides in honey.

Vichapong et al. (2015) developed a different approach, called in-coupled syringe assisted octanol-water partition microextraction (IS-DLLME), for the determination of neonicotinoids in honey. A low-density solvent was used as an extraction solvent and two coupled syringes were used to increase dispersion of the extraction solvent into aqueous solution. The developed method showed limits of detection of 0.25-0.5 ng/mL, which are below the acceptable MRLs for neonicotinoids.

Considering the studies presented above, it can be concluded that DLLME, modified or not, proved to be simple and rapid. Also, it demands low solvent use, it has high factor enrichment, good linearity in the concentration

range studied and low cost, making this technique suitable for pesticides analysis in complex samples such as honey.

3.1.5.2. Microextraction by packed sorbent

Microextraction by packed sorbent (MEPS), developed by Abdel-Rehim in 2004, is the miniaturization of conventional SPE (ABDEL-REHIM, 2004). MEPS can be used in two different ways: the sorbent is packed into the syringe and it is held between two filters of polypropylene or the sorbent can be accommodated in a small container situated between the syringe body and the needle. MEPS can easily be used with GC or LC without the need to modify the extraction device (ABDEL-REHIM et al., 2008; VIANA et al., 2014). The selection of the sorbent, made according to the properties of the analyte, is essential to achieve acceptable levels of purification. The phases normally used are silica chemically bound to C2, C8 and C18, benzene sulfonic acid cation exchange, polymers (polystyrene particles), molecularly imprinted polymers (MIP) and monolithic organic sorbents (BLOMBERG, 2009).

MEPS was applied in the analysis of pesticides in honey by Salami and Queiroz (2013). The method involved MEPS and gas chromatography coupled to mass spectrometry (MEPS–GC–MS) for the multi-residue analysis of 22 pesticides. The recoveries (82-114%) were adequate for all analytes. The developed method showed the following advantages: the sorbent was reused more than 40 times with minimum loss of extraction efficiency and the extraction time was reduced (approximately 4 min). Moreover, the amount of honey sample (3.0 g) and organic solvent (desorption, 20 mL of ethyl acetate) were low. However, due to the complexity of the honey matrix, LLE was necessary before MEPS, which is not desirable as it prolongs the time of the analysis.

3.1.5.3. Solid phase microextraction

Solid phase microextraction (SPME) was developed by Pawliszyn and collaborators in 1990 and combines sampling and pre-concentration in a single step (ARTHUR & PAWLISZYN, 1990). It is performed by immersion of a silica fiber coated with a stationary phase in an aqueous sample. Campillo et al.

(2012) developed an environmentally friendly method for the determination of seven organotin compounds (OTCs) in honey using headspace solid-phase microextraction (HS-SPME). The analytes were derivatized with sodium tetraethylborate ($NaBEt_4$) and the derivatization and preconcentration steps were optimized. Optimization of the SPME method was carried out using 1 g of honey, previously fortified, dissolved in 10 mL of pH 5 buffer. The time and temperature during extraction were 15 min at 80 ^{o}C, and the desorbed compounds were analyzed using GC. Six fiber coatings of different polarities were tested. A 100-µm polydimethylsiloxane fiber was the most suitable for the preconcentration of the derivatized analytes from the headspace of an aqueous solution containing the sample. The headspace mode was selected not only because higher responses were obtained for all compounds, but also because reproducibility and fiber-life time were higher.

Monolithic material has been recently applied to SPME. Zhang et al. (2015) used a porous poly(methacrylic acid-co-ethylene dimethacrylate) monolithic fiber for SPME of benzimidazole anthelmintics in honey. Under optimum experimental conditions, the limits of detection of the method varied from 0.086 to 0.28 µg/L.

In the study developed by Zali et al. (2015) electrospun polystyrene nanostructure was used as coating material on a stainless steel wire for SPME. Experimental parameters which affect the SPME method, such as extraction temperature and time, ionic strength and desorption were investigated and optimized. The final conditions were: extraction time of 10 min, extraction temperature of 70 ^{o}C, desorption temperature of 250 ^{o}C, desorption time of 5 min, NaCl 2.5% (w/v), and agitation at 250 rpm. All the experiments were performed in natural pH of honey (3.2 – 4.5). Polystyrene nanofibers were useful in simultaneous extraction of trace amounts of seven pesticides with different polarities from honey samples. The limits of detection ranged from 0.1 to 0.2 µg/L. Each fiber could be used at least 100 times without significant physical damages or loss of extraction efficiency.

3.1.5.4. Stir bar sorptive extraction

Stir bar sorptive extraction (SBSE) is accomplished by stirring the sample with a stir bar covered with a sorbent, generally poly(dimethylsiloxane) (PDMS), for a given time. Analyte enrichment occurs by partitioning between the polymer and the aqueous phase according to their distribution constant and its desorption takes place by means of temperature in the injector (GC) or by liquid removal (LC) (BALTUSSEN et al., 2002). SBSE was observed to be a good alternative due to its advantages of high recovery, good reproducibility and convenience (YU & HU, 2009).

Yu and Hu (2009) optimized a SBSE method for the extraction of organophosphorus pesticides (OPPs) in honey. The effect of extraction time in the range of 5 - 25 min was examined fixing the stirring rate at 700 rpm. The effect of temperature (ranging from 15 to 50 ^{o}C) on extraction efficiency was also investigated by using a water bath. Sodium chloride concentrations varied from 0 to 25% m/v, and the results indicated that the extraction efficiency of the five OPPs increased with the amount of salt. However, increasing the stirring rate improved extraction efficiency of OPPs in honey. Desorption time was also tested in the range of 5-40 min by using 50 µL of acetonitrile as the desorption solvent. The effect of sample pH on the extraction of organophosphorus pesticides was studied in the range of 2 to 8 in spiked aqueous solutions. No effect of sample pH on the extraction efficiency of organophosphorus was observed.

3.1.5.5. Single-drop microextraction

Single-drop microextraction (SDME) is a technique that simultaneously extracts and preconcentrates the analytes. This microextraction technique employs a microdrop of organic, water-immiscible solvent suspended at the end of a microsyringe needle. This microdrop is immersed in a sample solution for a period of time when extraction takes place (JEANNOT & CANTWELL, 1997; TSIROPOULOS & AMVRAZI, 2011).

The dilution of the sample, the volume, the pH, the ionic strength (NaCl %) and the stirring rate of the donor solution, as well as the duration of

extraction (time) and the depth of the drop in the stirring solution (drop depth), are the main variables that affect the SDME of pesticides in honey. These parameters were evaluated by Tsiropoulos and Amvrazi (2011), who optimized the method for multiclass pesticides in honey. The developed method was successfully applied obtaining recoveries in the range of 70.8% for captan to 120% for fenarimol.

3.1.5.6. Magnetic solid phase extraction

Magnetic solid-phase extraction (MSPE) was developed by Safarikova and Safarik (1999) and is based on the use of magnetic or magnetizable adsorbents. In MSPE the magnetic adsorbent, added to a solution or suspension, extracts the analyte. Afterwards, an appropriate magnetic separator is used to recover the adsorbent with the adsorbed analyte. Then, the analyte is eluted from the adsorbent and analyzed. The sorbents generally used in MSPE are magnetic nanoparticles (MNPs), generally Fe_3O_4 or γ-Fe_2O_3 (SOUZA et al., 2014). However, MNPs are difficult to disperse in aqueous samples, which may alter their stability and lose their adsorption ability in complex matrices. Compared to traditional SPE sorbents, MNPs possess high surface area and have unique magnetic properties (YE et al., 2012).

In the method proposed by Du et al. (2013), based on magnetic cobalt ferrite filled carbon nanotubes (MFCNTs), 5 g of honey spiked with standard solutions of organochlorine pesticides was dissolved in 100 mL of water to obtain final concentrations of 5.0 µg/L. A volume (25 mL) of every spiked sample was mixed and extracted with 10 mg of MFCNTs in a conical flask. The flask was stirred at 120 rpm for 40 min. The MFCNTs were separated from the solution by a magnet, dried and resuspended in 200 µL of ethyl acetate. The vial was kept in an ultrasonic washer for 15 min to desorb the OCPs on the MFCNTs. The suspension was separated with a magnet and 2 µL of aliquot were injected into the GC-ECD for analysis. After optimization, the best conditions were: water as solvent, oscillatory frequency of 120 rpm, extraction time of 40 min, ethyl acetate as desorption solvent, 200 µL of desorption solvent volume and 15 min of desorption time. Recoveries were in the range of 83.2 - 128.7%.

3.2. Separation and detection

Several analytical methods have been used to separate and detect pesticides in honey. Due to the low concentration of these compounds and the high complexity of the matrix, the use of analytical techniques which provides high selectivity and sensitivity is essential. GC–EI–MS and LC–ESI–MS/MS are the most widely used techniques for multi-residue analysis of pesticides in bee products (WIEST et al., 2011). The choice of the separation technique depends mostly on the characteristics of the pesticides of interest. The volatile, semi-volatile and thermally stable compounds can be determined by GC, whereas non-volatile and/or thermally unstable ones should be determined by LC (KUJAWSKI et al., 2014).

Studies of pesticides in honey reported in the literature between 2008 and 2015 are summarized in Tables 5 and 6. All of them used chromatography, either gas or liquid, to separate these compounds.

Table 5. Methods of separation and detection for pesticides determination in honey by gas chromatography

Classes of pesticides analyzed	Column	Detection	LOD (µg/kg)	LOQ (µg/kg)	Reference
Organophosphorous, sulphite ester, benzilate, bridged diphenyl, pyrethroid (7 pesticides)	HP-5 MS (300 x 0.25 mm, 0.25 µm)	MS	0.1 - 2.0 µg/L	0.5 - 10 µg/L	ZALI et al., 2015
Organochlorines, pyrethroids (18 pesticides)	HP-5 MS (300 x 0.25 mm, 0.25 µm)	µECD	0.5 - 30	2 - 60	MALHAT et al., 2015
Organochlorines, organophosphorus, carbamates, pyrethroids, benzilate, pyrimidinol, chloronitrile, triazine phosphorothiolates, dicarboximides, dinitroaniline (46 pesticides)	ECD: PAS-5 tested Ultra 2 Silicon (250 x 0.32 mm, 0.52 µm) NPD: PAS-1701 tested 1701 Silicon (250 x 0.32 mm, 0.25 µm)	ECD NPD	1 - 168	nf	EISSA et al., 2014
Triazoles (5 pesticides)	BPX-5 - 5% phenyl methyl siloxane, 95% dimethyl siloxane (300 x 0.25 mm, 0.25 µm)	NPD	0.05 - 0.21	0.15 - 1.1	FARAJZADEH et al., 2014
Organophosphorus, chloroacetamide, strobilurin, phthalimide, pyrethroids, cyanoacetamide oxime, triazoles, organochlorines, sulphamide, morpholine, phenylamides, chlorinated hydrocarbon, dicarboximide, phosphorothiolate, anilinopyrimidine, sulphite ester, bridged diphenyl, benzimidazole (53 pesticides)	ECD: HP 5 (300 x 0.32 mm, 0.25 µm) NPD: HP 50 (300 x 0.32 mm,0.25 µm)	NPD and µECD	1 - 792	1 - 2,467	LOPEZ et al., 2014
Organophosphorus, organochlorines, pyrethroids, strobins, triazoles, chloronitrile, dinitroanilines, pyrazole (24 pesticides)	DB-5MS (300 x 0.25 mm, 0.25 µm)	ECD	3 - 6	10 - 20	ORSO et al., 2014
Organochlorines, chlorinated hydrocarbon, pyrimidinol, organophosphorus, triazole, phthalimide, quinoline, phenylpyridinamine, strobilurin, dicarboximide, carboxamide, anthranilic diamide, tetronic acid (28 pesticides)	Rt-5MS - 5% diphenyl, 95% dimethylpolysiloxane (350 x 0.25 mm, 0.25 µm)	MS/MS	0.75 - 1.48	2.25 - 4.44	PANSERI et al., 2014

Table 5. Methods of separation and detection for pesticides determination in honey by gas chromatography (continuation…)

Classes of pesticides analyzed	Column	Detection	LOD (µg/kg)	LOQ (µg/kg)	Reference
Organochlorines (8 pesticides)	AE SE-54 (300 x 0.25 mm, 0.33 µm)	ECD	0.0013 - 0.0036 (µg/L)	nf	DU et al., 2013
Organochlorines, organophosphorus, carbamates, pyrethroids, dicarboximide, bridged diphenyl and others unclassified (22 pesticides)	NST-05MS - 5% phenyl Dimethylpolysiloxane (300 x 0.25 mm, 0.25 µm)	MS	nf	10	SALAMI & QUEIROZ, 2013
Organotins (7 pesticides)	GC-AED: HP-5 (250 x 0.32 mm, 0.17 µm) GC-MS: HP-5MS (300 x 0.25 mm, 0.25 µm)	MS and AED	GC–AED: 0.002–0.022 GC–MS: 0.0003 - 0.0043	nf	CAMPILLO et al., 2012
Organonitrogens (9 pesticides)	GC-NPD: ZB-5 column (300 x 0.32 mm, 0.50 µm)	NPD and MS	GC-NPD: 0.36 - 1.75 GC-MS: 0.13 - 0.25	nf	CHIENTHAVORN et al., 2012
Organochlorines (11 pesticides)	Zebron ZB 5-MS (300 x 0.25 mm, 0.25 µm)	MS	0.1 - 4	0.3 - 13.2	KUJAWSKI et al., 2012
Organochlorines (15 pesticides)	AT-5MS - 5%-phenyl, 95% methoxlpolysiloxane (300 x 0.25 mm, 0.25 µm)	ECD and IT/MS	GC-ECD: 0.4 - 3.0 GC-IT/MS: 0.2 - 4.0	GC-ECD: 1.4 - 10 GC-IT/MS: 0.8 - 13	ZACHARIS et al., 2012
Pyrethroid (1 pesticide)	HP 5 (300 x 0.25 mm, 0.25 µm)	MS	0.1	2.5	BONZINI et al., 2011
Organochlorines, chlorinated hydrocarbon, organophosphorus, triazines, benzonitrile (18 pesticides)	HP-5MS (300 x 0.25 mm, 0.25 µm)	MS	1	nf	MUJIC et al., 2011
Organophosphorous, organochlorines, chlorinated hydrocarbon, phthalimide, dicarboximide, benzilate, pyrimidine (14 pesticides)	HP-5MS (300 x 0.25 mm, 0.25 µm)	ECD and MS	0.01 - 3.75	0.03 - 10.6	TSIROPOULOS & AMVRAZI, 2011

Table 5. Methods of separation and detection for pesticides determination in honey by gas chromatography (continuation…)

Classes of pesticides analyzed	Column	Detection	LOD (µg/kg)	LOQ (µg/kg)	Reference
Organochlorines, amide, organophosphorus, pyrethroids, triazoles, pyrimidines, dicarboximide, synergist, insect growth regulator (80 pesticides)	DB-XLB (300 x 0.25 mm, 0.25 µm)	ToF-MS	0.1 - 23.9	3.0 - 65.8	WIEST et al., 2011
Organophosphorus, organochlorines, pyrethroids, n-methyl-carbamates, bromopropylate (53 pesticides)	GC-FPD: RTX-1701 (300 x 0.25 mm, 0.25 µm) GC-MS: HP-5MS (300 x 0.25 mm, 0.25 µm)	FPD and MS	0.2 - 8	0.5 - 25	AMENDOLA et al., 2011
Organophosphorus (4 pesticides)	VF-5MS (250 x 0.25 mm, 0.25 µm)	MS	0.03 - 0.47	nf	FONTANA et al., 2010
Organophosphorus, pyrethroids (4 pesticides)	HP-5 - 5% phenyl – 95% dimethyl-siloxane (300 x 0.25 mm, 0.1 µm)	ECD	14 - 16	28 - 33	PINHO et al., 2010
Organochlorines (11 pesticides)	DB-5MS (300 x 0.25 mm, 0.25 µm)	IT/MS	0.001 - 0.010	nf	WANG et al., 2010
Organochlorines (24 pesticides)	DB-5ms capillary column (300 x 0.25 mm, 0.25 µm)	ECD	0.4 - 2	1 - 7	YAVUZ et al. (2010)]
Pyrethroids, organochlorines, organophosphorus (8 pesticides)	HP-1 (250 x 0.53 mm, 1 µm)	ECD	0.001 - 0.05	0.05 - 1.0	MUKHERJEE, 2009
Organophosphorus (5 pesticides)	HP-5 (300 x 0.32 mm, 0.25 µm)	FPD	0.013 - 0.081 (µg/L)	nf	YU & HU, 2009
Organophosphorus (10 pesticides)	RTX-5 (300 x 0.53-mm, 1.0 µm)	NPD	nf	0.02 - 0.54	BALAYIANNIS & BALAYIANNIS, 2008

Table 5. Methods of separation and detection for pesticides determination in honey by gas chromatography (continuation…)

Classes of pesticides analyzed	Column	Detection	LOD (µg/kg)	LOQ (µg/kg)	Reference
Organochlorine, cyclodiene, pyrethroids, organophosphorus (20 pesticides)	BP225 - 50 % cyanopropyl + 50 % diethyl siloxane) (500 x 0.53 mm, 0.5 µm)	ECD	0.05 - 5.0	nf	CHOUDHARY & SHARMA, 2008

Legend: AED = atomic electron detector; ECD = electron capture detector; FPD = flame photometric detector); IT = ion trap; IT/MS = ion trap mass spectrometry; LOD = limit of detection; LOQ = limit of quantification; MS = mass spectrometry; MS/MS = tandem mass spectrometry; nf = not found; NPD = nitrogen phosphorus detector; ToF = time-of-flight.

Table 6. Methods of separation and detection for pesticides determination in honey by liquid chromatography

Classes of pesticides analyzed	Column	Mobile Phase	Detection	LOD (µg/kg)	LOQ (µg/kg)	Reference
Acylamino acid, benzofuran, anilinopyrimidines, carbamates, aryloxyphenoxypropionates, benzimidazoles, carbanilate, carboxamides, chloroacetamides, cyanoimidazole, diacylhydrazines, dicarboximides, dinitroaniline, hydroxyanilide, imidazoles, morpholines, neonicotinoids, organophosphorous, oxadiazine, phenylamides, phenylpyrazoles, phenylureas, phosphorothiolate, pyrazoles, pyrethroids, pyridines, pyridazinone, pyrimidines, ureas strobilurins, sulphite ester, triazines, tetrazine, tetronic acid, triazoles and others pesticides unclassified (116 pesticides)	C18 (100 x 2.0 mm, 2.2 µm)	Gradient elution Methanol: ammonium acetate 10mM	ESI/MS/MS positive mode	5.0	10.0 - 25.0	TETTE et al., 2016b

Table 6. Methods of separation and detection for pesticides determination in honey by liquid chromatography (continuation…)

Classes of pesticides analyzed	Column	Mobile Phase	Detection	LOD (µg/kg)	LOQ (µg/kg)	Reference
Amidines, diphenyl oxazoline, pyrazole, phenylpyrazole, carboxamide, sulphite ester, pyridazinone, tetronic acid (9 pesticides)	C18 (150 x 2.1 mm, 5 µm)	Gradient elution Methanol: ammonium formate 5mM	ESI/MS/MS positive and negative mode	nf	1.0	NAKAJIMA et al., 2015
Chlorophenols (6 pesticides)	C18 (150 x 4.6 mm, 5 µm)	Gradient elution Acetonitrile: water with 0.5% phosphoric acid	UV	0.8 - 3.2 (µg/L)	nf	FAN et al., 2015
Neonicotinoids (7 pesticides)	C18 (50 x 4.6 mm, 1.8 µm)	Gradiente elution Acetonitrile:water with 0.2% formic acid	UV	1.5 - 2.5	5.0 - 10.0	JOVANOV et al., 2015
Organophosphorous (14 pesticides)	C18 (100 x 4.6 mm, 5 µm)	Gradient elution Water:Methanol	ESI/MS/MS positive mode	0.05 - 21.5	nf	NAGGAR et al., 2015
Neonicotinoids (7 pesticides)	C18 (150 x 4.6 mm, 5 µm)	Acetonitrile:water (1:3)	UV	0.25 - 0.50 (µg /L)	0.80 - 2.00 (µg/L)	VICHAPONG et al., 2015
Neonicotinoids, carbamates, amidine, triazines, strobilurins, carboxamide, benzimidazole, triazolone, amine, organophosphorous, triazoles, anilinopyrimidines, benzoylurea, morpholine, pyrethroids, oxadiazine, urea, imidazole, dicarboximide, phenylamide, diacylhydrazine, chloroacetamide, dinitroaniline, cyclic aromatic, tetronic acid, diacylhydrazine and others unclassified (55 pesticides)	C18 (150 x 2.1 mm, 5 µm)	Gradient elution Water: acetonitrile with 0.1% formic acid)	ESI-HRMS positive mode	0.1 - 10	nf	COTTON et al., 2014

Table 6. Methods of separation and detection for pesticides determination in honey by liquid chromatography (continuation…)

Classes of pesticides analyzed	Column	Mobile Phase	Detection	LOD (µg/kg)	LOQ (µg/kg)	Reference
Neonicotinoids, dinitroanilines, triazoles, organophosphorus, carbamates, dicarboximides (115 pesticides)	C18 (150 x 2.1 mm, 3.5 µm)	Gradient elution Water with 5 mM ammonium formate, 0.1% formic acid, and 0.02% acetonitrile. Methanol with 5 mM ammonium formate and 0.1% formic acid.	ESI/MS/MS positive and negative mode	0.03 - 23.3	0.1 - 78	KASIOTIS et al., 2014
Anilinopyrimidine, pyrazole, aryloxyphenoxypropionate, anilinopyrimidine, organophosphorus, strobilurin, pyridazinone, oxathiin, cyanoacetamide oxime, benzoylurea, morpholine, quinazoline, triazole, pyretroids, pyrazole, carbamate, chloroacetamide, triazine, thiocarbamate and others unclassified (30 pesticides)	C18 (50 x 2.1 mm, 1.7 µm)	Water: Methanol (90:10) with 10 mmol/L ammonium acetate Water: Methanol (10:90) with 10 mmol/L ammonium acetate	ESI-MS/MS positive mode	QuEChERS: 0.33 - 7.37 SLE: 0.35 - 7.09	QuEChERS: 1.10 - 23.65 SLE: 1.17 - 22.75	KUJAWSKI et al., 2014
Organophosphorus, carbamates (10 pesticides)	C18 (250 x 4.6 mm, 5 µm)	Acetonitrile:water (70:30)	UV	nf	nf	MONIRUZZA-MAN et al., 2014
Chloroacetamide, pyrazole, organophosphorus, pyrethroid, triazolone, neonicotinoid, oxadiazine, carbamate, strobilurin, phosphorothiolate, pyridazinone, aryloxyphenoxypropionate, and others unclassified (30 pesticides)	C18 (100 x 3 mm, 2.7 µm)	Gradient elution Water:methanol with10mM ammonium acetate	ESI/MS/MS positive mode	0.91 - 25	2.73 - 75	BARGANSKA et al., 2013
Neonicotinoids (7 pesticides)	C18 (50 x 4.6 mm, 1.8 µm)	Acetonitrile:water (20:80) with 0.1% formic acid	ESI/MS/MS positive mode	0.5 - 1.0	1.5 - 2.5	JOVANOV et al., 2013

Table 6. Methods of separation and detection for pesticides determination in honey by liquid chromatography (continuation…)

Classes of pesticides analyzed	Column	Mobile Phase	Detection	LOD (µg/kg)	LOQ (µg/kg)	Reference
Pyrethroids (4 pesticides)	C18 (250 x 4.6 mm, 5 µm)	Acetonitrile:water (83:17)	UV	0.03 - 0.05 (µg/L)	0.10 - 0.18 (µg/L)	LI et al., 2013
Alkylchlorophenoxy, phenylurea, phenylpyrazole (3 pesticides)	C18 (250 x 4.6 mm, 5 µm)	Gradient elution Methanol:water with phosphoric acid (1:1)	DAD ESI-MS/MS positive and negative mode	DAD: 16 - 100 MS/MS: 0,16 - 0,8	DAD: 50 - 312,5 MS/MS: 0.5 - 2.5	SAMPAIO et al., 2012
Amidine, benzimidazole, organophosohorus, phenylpyrazole, pyrethroid, neonicotinoid, triazole (8 pesticides)	C18 (50 x 3 mm, 3.5 µm)	Gradient elution Methanol:water with 0.1% formic acid	APCI-MS/MS positive and negative mode	1.6 - 330	5 - 1000	TOMASINI et al., 2012
Neonicotinoid, triazolone, pyridine, phenylamide, organophosphorus, imidazole, triazole, carbamate, sulfonylurea and other unclassified (13 pesticides)	C18 (100 x 2.1 mm, 3 µm)	Gradient elution Methanol:water with 0.1% formic acid	ESI-MS/MS positive mode	0.01 - 0.25	0.04 - 0.73	KUJAWSKI & NAMIESNIK, 2011
Amide, organophosphorus, carbamates, triazoles, nicotinoids, dicarboximide, imidazole, avermectins, formamidine, tetrazine, thiazolidine, synergist, insect growth regulator (80 pesticides)	C18 (50 x 2 mm, 1.8 µm)	Gradient elution Water with 0.3 mM ammonium formate and 0.05% formic acid and methanol	ESI/MS/MS positive mode	0.01 - 23.5	3.0 - 70.4	WIEST et al., 2011
Pyrethroids (4 pesticides)	C18 (250 x 4.6 mm, 5 µm)	Acetonitrile:water (70:30)	UV	0.21 - 0.38 (µg/L)	nf	ZHANG et al., 2011

Table 6. Methods of separation and detection for pesticides determination in honey by liquid chromatography (continuation…)

Classes of pesticides analyzed	Column	Mobile Phase	Detection	LOD (µg/kg)	LOQ (µg/kg)	Reference
Neonicotinoids, phenylpyrazoles (7 pesticides)	C8 (150 x 2.1mm, 3.5 µm)	Gradient elution Water with 0.15 % formic acid and 3 mM ammonium formate Methanol with 0.15 formic acid and 3 mM ammonium formate	ESI/MS/MS positive and negative mode	nf	nf	GARCÍA-CHAO et al., 2010
Organophosphorus, benzimidazole, amidine (4 pesticides)	C18 (50 x 2.1 mm, 5 µm)	Gradient elution Water:acetonitrile with 0.2% formic acid	ESI/MS/MS positive mode	0.2 - 3.5	0.6 - 6.1	DEBAYLE et al., 2008
Carbamates (3 pesticides)	C18 (150 x 2.1 mm, 3 µm)	Methanol:water (90:10)	ESI/MS/MS positive mode	20 - 40	50 - 130	ZHU et al., 2008
Ultra Performance Liquid Chromatography						
Neonicoticoids (6 pesticides)	(50 x 2.1 mm, 1.9 µm)	Gradient elution Water with 0.05% formic acid and 2mM ammonium formate Methanol with 0.05% formic acid and 2mM ammonium formate	ESI/MS/MS positive mode	nf	0.10 - 4	GALEANO et al., 2013

Table 6. Methods of separation and detection for pesticides determination in honey by liquid chromatography (continuation…)

Classes of pesticides analyzed	Column	Mobile Phase	Detection	LOD (µg/kg)	LOQ (µg/kg)	Reference
Organophosphorus, organochlorines, carbamates, pyrethroids, triazines, biopesticides, ureas, neocotinoids, triazoles (350 pesticides)	C18 (100 x 2.1 mm, 1.7 µm)	Gradient elution Water with 0.1% formic acid and 4 mM ammonium formate Methanol with 0.1% formic acid and 4 mM ammonium formate	Orbitrap/ESI/ MS positive and negative mode	25 - 50	nf	GOMEZ-PERÉZ et al., 2012
Neonicotinoids (12 pesticides)	C18 (100 x 2.1 mm, 1.8 µm)	Water:methanol (95:5) with 5mM ammonium formate and 0.1% formic acid Water:methanol (5:95) with 5mM ammonium formate and 0.1% formic acid	ESI/MS/MS positive mode	0.2 - 15	nf	KAMEL, 2010

Legend: APCI = atmospheric-pressure chemical ionization; ESI = electrospray ionization; FL = spectrofluorimetric detector; HRMS = high resolution mass spectrometry; LOD = limit of detection; LOQ = limit of quantification; MS = mass spectrometry; MS/MS = tandem mass spectrometry; nf = not found; QuEChERS (Quick, Easy, Cheap, Effective, Ruged and Safe); UV = Ultraviolet.

3.2.1. Gas chromatography

Gas chromatography (GC) coupled with quadrupole mass spectrometry detection is considered as a powerful technique for the quantitative determination of low levels of contaminants in complex matrices (KOLBERG et al., 2011). In this sense, it has been used extensively for the determination of pesticide in honey.

GC has been combined with different detection systems for the analysis of honey, including: (i) MS (AMENDOLA et al., 2011; ZACHARIS et al., 2012; SALAMI & QUEIROZ, 2013); (ii) MS/MS (PANSERI et al., 2014); (iii) NPD (FARAJZADEH et al., 2014; LOPEZ et al., 2014); (iv) ECD (DU et al., 2013; MALHAT et al., 2015); (v) AED (CAMPILLO et al., 2012); and (vi) FPD (YU & Hu, 2009; AMENDOLA et al., 2011). Mass spectrometry represents the most selective detector for pesticides and it provides structural information allowing unequivocal confirmation, which is of great importance in a multi-residue analysis. MS allows simultaneous detection and identification of co-eluting compounds, in which specific ions or their transitions are selected (KUJAWSKI & NAMIESNIK, 2011). Mass spectrometry is widely used for the detection of various groups of pesticides in honey. However, the successful confirmation of the analytes is dependent on the method chosen. The selected-ion monitoring mode (GC-SIM/MS) is used in most methods and has improved sensitivity due to its identification capability. In this mode, the three most intense ions of the analyte under study are selected; one ion is used for quantification and the other two are used for confirmation. More than two ions could be selected for confirmation, but this may compromise the sensitivity of the method. In the full-scan mode, all ions produced in the MS are used for the confirmation and quantification of the target analyte. The reliability of the identification of compounds can be achieved by standard MS libraries, such as the National Institute of Standards and Technology (NIST) library that contains more than 150,000 mass spectra of standard organic compounds (TAHBOUB et al., 2006).

Although GC is often reported as the most powerful separation tool, it requires a derivatization step for nonvolatile and for thermally unstable compounds. This introduces additional handling and reaction, which can

potentially reduce reproducibility and recovery rates (FERNANDEZ & PICO, 2002; PIRARD et al., 2007).

The choice of the GC column is a very important task in pesticide analysis. The stationary phase should be selected as a function of the polarity of the pesticides. Non-polar columns (5% phenyl 95% dimethylpolysiloxane) are the most commonly used for pesticide analysis in honey, as indicated in Table 5 (PINHO et al., 2010; CHIENTHAVORN et al., 2012; PANSERI et al., 2014). However, medium-polar (50% phenyl 50% dimethylpolysiloxane) and medium to high-polar columns (50% Cyanopropylphenyl Polysiloxane) have also been used (CHOUDHARY & SHARMA, 2008; MUKHERJEE, 2009). Moreover, other column parameters such as length, inner diameter or film thickness can be optimized as a function of the number of pesticides that must be determined simultaneously.

In the recent past, GC-MS was the most widely used technique to analyze residues in foodstuffs compared to LC-MS (FARRE et al., 2014). HPLC was applied most successfully to the analysis of thermally unstable pesticides. However, the pesticides used today are more polar, thermally unstable or not easily vaporized, which allows the determination of these compounds by liquid chromatography (ALBERO et al., 2004; WILLE et al., 2011).

3.2.2. Liquid chromatography

Liquid chromatography (LC) has also been widely used to analyze pesticides in honey, especially for thermally labile compounds. Although mass spectrometry is the most useful and suitable detector for this kind of analysis (BARGANSKA et al., 2013; JOVANOV et al., 2013; NAGGAR et al., 2015), other types of detectors have also been used including variable wavelength (LI et al., 2013), diode array (DAD) (SAMPAIO et al., 2012; VICHAPONG et al., 2015) and spectrofluorimetric (AMENDOLA et al., 2011). However, these other types of detectors are generally used to analyze only few pesticides or few classes of pesticides.

Tandem mass spectrometry coupled with chromatography allows detection of pesticides at low concentrations in complex matrices. This system

improves detectability, reduces matrix interference and adds structural information. With multiple reactions monitoring (MRM) mode, the spectrometer analyzes only the ions of interest, increasing reliability of the results (SAMPAIO et al., 2012). Therefore, LC–MS/MS methods have been widely developed in the last years to evaluate the presence of target analytes in commercial honey and related samples. In terms of stationary phase, C18 column (4.6 and 2.1 mm i.d.) is almost consensus for the separation of pesticides (DEBAYLE et al., 2008; GOMEZ-PERÉZ et al., 2012; TOMASINI et al., 2012).

Ultra high-performance liquid chromatography (UHPLC-MS/MS) has been used for several pesticide classes in honey. Gómez and Pérez (2012), using ultra-high performance liquid chromatography coupled to high resolution Orbitrap mass spectrometry (UHPLC–Orbitrap-MS), developed a method to evaluate several classes of contaminants (pesticides, biopesticides and veterinary drugs), a total of 350 compounds, with a run time of only 14 min.

However, despite all the advantages provided by the current detection techniques, an appropriate sample preparation method is still indispensable, since matrix effects can significantly impact detection by forming significant noise and changing ionization efficiency with consequent decrease in sensitivity (KUJAWSKI & NAMIESNIK, 2011).

3.2.3. Matrix effect

The determination of pesticide residues in honey is usually challenging due to their very low concentrations and the interference of the complex matrix (KASIOTIS et al., 2014). Matrix effect can result in an enhanced or decreased analyte signal from extracts obtained in the presence of matrix compared to those obtained in solvent (FARAJZADEH et al., 2014).

Usually, the study of matrix effect is accomplished by two different methods. One option is to compare the slopes of standards in solvent with the slopes of matrix-matched standards. Matrix effect (%) is calculated with the equation [(slope of standards in matrix - slope of standards in solvent/ slope of standards in solvent) x 100]. When the obtained values are in the range of -20% to +20% matrix effect is considered low; when they are between -50% and -

20% or +20% and +50% it is considered medium; and if these values are lower than -50% or higher than +50% matrix effect is considered high (TOMASINI et al., 2012). Another approach is the comparison of the areas of standard solution prepared in matrix and standard solution prepared in solvent multiplied by 100. When the result is equal to 100%, no matrix effect is present, while values higher or lower than 100% indicate suppression or enrichment of the ionization by the matrix components (SAMPAIO et al., 2012).

Honey extract consists basically of carbohydrates, such as glucose and fructose, which can induce matrix effect (ORSO et al., 2014). Orso et al. (2014) developed a modified QuEChERS method coupled to gas chromatography with electron capture detection (GC-ECD) and observed a negative matrix effect for chlorpyrifos ethyl (-34%), endosulfan beta (-44%), endosulfan sulfate (-74%), 4,4-DDE (-54%), dieldrin (-46%), endrin II (-73%) and tetradifon (-31%). On the other hand, positive matrix effect was observed for chlorpyrifos methyl (+109%), chlorothalonil (+20%), malathion (+117%), lambda-cyhalothrin (+171%), bifenthrin (+133%), bromophos methyl (+341%), endosulfan alpha (+19%), endrin I (+75%), fipronil (+78%), heptachlor (+29%), heptachlor epoxide (+36%) hexachlorobenzene (+80%), lindane (+140%), trifloxystrobin (+31%), trifluralin (+23%), kresoxim methyl (<10%) and trichlorfon (<10%). In order to reduce the matrix effect observed the authors prepared the analytical curves in the matrix.

Concentration of honey extract increases the analyte concentration and signal. On the other hand concentration can also increase matrix effects and cause ion suppression in the ionisation source resulting in poorer signals with higher noise. Therefore, an alternative approach to reduce matrix effect is the dilution of the extracts (KUJAWSKI et al., 2014) and was the strategy used by Blasco et al. (2004). Recoveries were higher with diluted samples.

Studies have shown that matrix effect depends on the floral origin of honey samples and the use of matrix-matched standards is needed to minimize the quantitative errors arising from the matrix effects (TOMASINI et al., 2012).

4. Conclusion and outlook

The determination of pesticide residues in honey is necessary for ensuring that human exposure to contaminants, especially by dietary intake, does not exceed acceptable levels for health. Furthermore, it is an important tool for the determination of environmental contamination by pesticides. Accordingly, several methods have been developed in the last years to determine residues of pesticides in honey by means of highly sensitive methods.

When comparing the review published in 2007 to the present review, contrary to what was expected by the author, who believed that SBSE and MALDI-TOF-MS would be widely used, the trend to sample preparation for pesticide analysis in honey is the use of QuEChERS and miniaturized techniques such as DLLME, MEPS and SPME. The main advantages of these approaches are the use of less solvents, speed and low cost of analysis. These techniques are expected to have the most pronounced development in the near future.

Regarding the analytical techniques, UHPLC and tandem mass spectrometry tend to be the ideal technique because of the requirements of regulatory agencies that impose even lower limits. These detectors meet these requirements in the sense of high selectivity and sensitivity, and accurate identification of the analytes at very low detection limits. Furthermore, a larger number of pesticides can be analyzed simultaneously.

Another noticeable trend is the development of multiresidue methods. Some studies have described the simultaneous analysis of more than one hundred pesticides from several chemical classes. It has been possible due to the development of instruments with high selectivity and sensitivity.

CAPÍTULO II - MULTICLASS METHOD FOR PESTICIDES QUANTIFICATION IN HONEY BY MEANS OF MODIFIED QUECHERS AND UHPLC-MS/MS

Abstract

Bee products can be produced in an environment contaminated by pesticides that can be transported by honey bees to the hive and incorporated into the honey. Therefore, rapid and modern methods to determine pesticide residues in honey samples are essential to guarantee consumers' health. In this study, a simple multiresidue method for the quantification of 116 pesticides in honey is proposed. It involves the use of a modified QuEChERS procedure followed by UHPLC-MS/MS analysis. The method was validated according to the European Union SANCO/12571/2013 guidelines. Acceptable values were obtained for the following parameters: linearity, limit of detection (0.005 mg/kg) and limit of quantification (0.010 and 0.025 mg/kg), trueness (for the four tested levels the recovery assays values were between 70 and 120%), intermediate precision (RSD<20.0%) and measurement uncertainty tests (<50.0%). The validated method was applied for determination of 100 honey samples from five states of Brazil.

Keywords: pesticides; honey; QuEChERS; UHPLC-MS/MS; proficiency test

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1. Introduction

Honey is one of the most used products of the hive, both naturally and in several industrialized forms (KOMATSU et al., 2002). Known since ancient times, honey has always attracted the attention of man, especially because of its sweet taste (ROSSI et al., 1999; BERA & ALMEIDA-MURADIAN, 2007). Furthermore, several hive products have been appreciated due to their antimicrobial and antiseptic properties. However, in recent years, the pesticide monitoring in honey has become a public health issue in view of the growth of the levels of these chemicals in bee products (RIAL-OTERO et al., 2007; LI et al., 2013). Therefore, the monitoring of pesticide residues in honey is important to evaluate the potential risk of these products to consumers' health. Also, such monitoring can provide information about the use of pesticides in crop fields around the hives and in their neighborhoods. In this case, honey can be used as a bio-indicator for the evaluation of environmental impact (RISSATO et al., 2006).

In this context, analytical methods for the determination of pesticides in honey must be available for routine analysis. The determination of pesticide residues in foods requires a prior step of sample preparation due to the low concentrations of the analytes in the sample, the distinct chemical properties of the analytes and the complexity of the matrices (PRESTES et al., 2009). Although most of these procedures are carried out by conventional techniques, such methods are generally not applicable to all food matrices, do not produce clean extracts and generate low recovery. These disadvantages have led to the development of new approaches with an emphasis on the practicality of implementation, the use of significantly lower amounts of organic solvents, and the ability to detect analytes in very low concentrations. In recent years, efforts in the field of analytical chemistry focused on the miniaturization of sample preparation associated with improvement in selectivity and sensitivity (MELWANKI & FUH, 2008). However, these efforts are far from being considered ideal, due to the limitation of application, quickness, sensitivity and reliability of the results (MARTÍNEZ-VIDAL et al., 2005). In this context, QuEChERS (an acronym for quick, easy, cheap, effective, rugged, and safe),

developed by Anastassiades et al. (2003), is an appropriate alternative. This technique, which has the advantages of being fast, easy, economical, effective, robust and secure, can be applied in any laboratory, due to the simplification of the steps (PRESTES et al., 2009). This approach has become popular for sample preparation at the international level (CIESLIK et al., 2011).

Besides the extraction and purification procedure, the choice of appropriate separation and detection techniques is a step of fundamental importance. Technological advances in mass spectrometry technique allow meeting the criteria of sensitivity and selectivity (CHIARADIA et al., 2008). Accordingly, the performance of liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) has shown great success in multiresidue pesticide analysis in complex food matrices such as honey (LOPEZ et al., 2008; WIEST et al., 2011; TOMASINI et al., 2012; BARGANSKA et al., 2013; JOVANOV et al., 2013; Orso et al., 2016). This technique provides information regarding the characteristic ion of each analyte as well as two or more transitions of these ions, useful to quantify and confirm the analytes at concentrations consistent with maximum residue levels (MRLs) established (Martins Júnior et al., 2006).

Several studies on multiresidue determination of pesticides in honey have been reported in the literature. Kasiotis et al. (2014) developed a method to investigate the occurrence of 115 pesticides of different chemical classes such as neonicotinoids, organophosphates, triazoles, carbamates, dicarboximides and dinitroanilines in honey from different areas of Greece using modifications of the QuEChERS technique and LC-MS/MS. The total chromatographic run time was 35 min. Similarly, the method developed by Cotton et al. (2014) evaluated the occurrence of 83 pesticides and antibiotics of different classes in honey from France using QuEChERS and LC-MS/MS in a run time of 30 min. Kujawski et al. (2014) determined pesticides in honey after 14 min run using two extraction techniques, QuEChERS and extraction on a diatomaceous earth support (SLE). However, the developed method was applied to only 30 pesticides including acaricides, insecticides, herbicides and fungicides. Rapid methods for multiresidue analysis of pesticides in honey have not been described in the literature. Gómez-Pérez et al. (2012) created a

method for the simultaneous analysis of more than 350 pesticides and veterinary drugs in honey using ultra-high performance liquid chromatography coupled to high resolution Orbitrap mass spectrometry (UHPLC-Orbitrap-MS) in a run time of 14 min, but the liquid liquid extraction was time consuming, due to the 1 hour agitation required for the extraction of the compounds.

Therefore, the aim of this study was to develop and validate a rapid, sensitive and selective method for determination of 116 pesticide residues from 35 different classes (acylamino acid, anilinopyrimidine, aryloxyphenoxypropionate, benzimidazole, benzofuran, carbamate, carbanilate, carboxamide, chloroacetamide, cyanoimidazole, diacylhydrazine, dicarboximide, dinitroaniline, hydroxyanilide, imidazole, morpholine, neonicotinoid, organophosphate, oxadiazine, phenylamide, phenylpyrazole, phenylurea, phosphorothiolate, pyrazole, pyrethroid, pyridazinone, pyridine, pyrimidine, strobilurin, sulphite ester, tetrazine, tetronic acid, triazine, triazole, urea and other pesticides unclassified) in honey using QuEChERS and ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS). The developed method was validated according to European Union SANCO/12571/2013 guideline (SANCO, 2013). Also, measurement uncertainty was evaluated as well as method performance by means of participation in a proficiency test. Finally, the method was used to evaluate the quality of the honey produced in five states from Brazil.

2. Experimental

2.1. Material

2.1.1. Honey samples

Honey samples were purchased from consumer stores or provided by honey producers or cooperatives: 66 from the state of Minas Gerais (49 wild flower honey, 4 from eucalyptus, 1 from *Vernonia polyanthes* and 12 without the flower type), 9 samples from São Paulo (1 wild flower honey and 8 without the flower type), 18 samples from Santa Catarina (all wild flower honey), 2 samples

from Espírito Santo (all wild flower honey) and 5 from Pará (all wild flower honey). All collected samples were produced by *Apis mellifera* honey bees except one sample from Pará, which was produced by *Melipona scutellaris*. The blank honey samples were acquired from the consumer market. The samples were stored at ambient temperature (20 ^{o}C) until analysis. Honey sample from the provider BIPEA, code 18-3619-0038, analyzed in the proficiency test, was maintained under refrigeration (5 ^{o}C) until analysis.

2.1.2. Chemicals and reagents

Acetonitrile and glacial acetic acid were supplied by Merck (Darmstadt, Germany), methanol, ethyl acetate and formic acid were obtained from Tedia (Ohio, USA), all HPLC grade. Polymerically bonded ethylenediamine-N-propyl phase (PSA) (Varian, Palo Alto, CA, USA), anhydrous magnesium sulfate (purity ≥ 97% - Sigma-Aldrich, Saint Louis, MO, USA), Florisil (Mallinckrodt, St. Louis, USA), and anhydrous ammonium acetate and sodium acetate (Vetec - Rio de Janeiro, RJ, Brazil) were of analytical grade. The solutions were prepared with ultra pure-water (Milli-Q Plus system; Millipore Corp., Billerica, MA, USA). All the reference standards were of high purity grade (>98.0%) and were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Individual stock solutions were prepared at 1000 mg/L in acetonitrile or methanol and stored in a freezer at -18 ^{o}C. The working solutions were prepared through appropriate dilutions of the stock solutions.

2.2. Apparatus

2.2.1. Chromatography parameters

The UHPLC system (Shimadzu LC20ADXR) comprised a binary pump (Shimadzu LC20ADXR), an auto sampler (Shimadzu SIL20ACXR) and a column oven (Shimadzu CTO20AC). Chromatography was carried out using a Shim-pack XR-ODSII column (2.0 x 100 mm, 2.2 µm particle size) with a mobile phase consisting of ammonium acetate (10 mmol/L) (phase A) and methanol

(phase B) both acidified with 0.1% formic acid at a flow rate of 0.5 mL/min. The gradient elution program was as follows: 0 min, 50% B; 6 min, 80% B; 10 min, 90% B; 10.5 min, 50% B; 10.5-13 min, 50% B. The total chromatographic run time was 13 min. Injection volume was 5 µL and the column temperature was set at 60 ^{o}C. The chromatographic method was previously developed by Madureira et al. (2012) and was adapted for the UHPLC system.

2.2.2. Mass spectrometry parameters

Mass spectrometry analysis was performed using a 5500 Triple Quadrupole mass spectrometer (Applied Biosystems, MDS SCIEX, Ontario, Canada). The instrument was operated using electrospray ionization (ESI) in the positive ion mode. Instrument settings, data acquisitions and processing were controlled by the software Analyst (Version 1.5.1, Applied Biosystems). Source parameters were optimized as follows: ion spray voltage 4.5 kV for ESI (+), curtain gas 20 psi, collision gas 8 psi, nebulizer gas and auxiliary gas 30 psi and ion source temperature 500 ^{o}C. Retention time, precursor ion, transitions, collision energy potentials (CE) and collision exit potentials (CXP) and optimal declustering potential (DP) of all studied analytes are shown in Table 1. Two SRM transitions were used for each analyte, one for quantification and the other for qualification to avoid false negatives at trace pesticide levels.

Table 1. Retention time windows (RTWs) and MS/MS conditions for each compound

Compound	RTWs (min)	Quantification transition (CE[a]; V; CXP[b]; V)	Confirmation transition (CE[a]; V; CXP[b]; V)	DP[c] (V)
3-hydroxy carbofuran	0.76-0.80	238.1 < 163.1 (21; 4)	238.1 > 181.2 (15; 2)	82
Acetamipride	0.74-0.78	223.1 > 126.0 (29; 12)	223.1 > 73.0 (71; 8)	51
Alachlor	5.55-5.75	270.1/272.1 > 238 (15; 22)	270.1/272.1 > 162.1/240.0 (27; 14/ 15; 22)	76/71
Aldicarb	1.18-1.25	208;1 > 116.0 (11; 3)	208;1 > 88.9 (20; 3)	51
Allethrin	7.99-8.41	303;1 > 135.1 (17; 12)	303;1 > 91.1 (55; 8)	106
Ametryn	4.20-4.40	228.0 > 186.0 (25; 16)	228.0 > 116.0 (35; 10)	71
Azinphos ethyl	5.07-5.33	346.0 > 132.2 (23; 12)	346.0 > 160.2 (15; 12)	76
Azinphos methyl	3.34-3.52	318.1 > 132.1 (23; 12)	318.1 > 261.1/160.0 (9; 24/11/16)	106
Azoxystrobin	3.99-4.20	404.1 > 371.9 (21; 34)	404.1 > 343.9 (29; 34)	101
Benalaxyl	6.21-6.52	326.0 > 148.0 (31; 12)	326.0 > 294.0 (15; 28)	81
Bitertanol	6.53-6.87	338.1 > 269.1 (13; 24)	338.1 > 99.0 (21; 10)	51
Buprofezin	8.15-8.30	306.2 > 201.1 (17; 18)	306.2 > 116.0 (21; 10)	56
Cadusafos	7.17-7.30	271.1 > 159.0 (19; 18)	271.1 > 215.0 (13; 10)	76
Carbaryl	1.95-2.05	202.2 > 145.1 (15; 14)	202.2 > 127.1 (39; 12)	66
Carbendazin	0.95-1.00	192.0 > 160.1 (25; 14)	192.0 > 132.1 (41; 12)	56
Chlorbupham*	3.86-4.06	241.1 > 172.0 (17; 16)	241.1 > 154.0 (29; 14)	51
Chlorfentezine	6.82-6.97	303.0 > 137.9 (21; 12)	303.0 > 102.0 (53; 8)	21
Chlorpyrifos-methyl	6.77-7.12	321.9 > 125.0 (27; 12)	321.9 > 289.8 (23; 26)	106
Chlortiophos	8.80-8.92	361.0 > 304.8 (23; 28)	361.0 > 192.0 (39; 16)	86
Cinidon-ethyl*	7.68-8.10	410.9 > 347.9 (31; 32)	410.9 > 365.9 (25; 34)	51
Cyazofamid	5.25-5.52	324.9 > 108.0 (19; 10)	324.9 > 261.0 (13; 24)	66
Cyhalofop butyl*	7.42-7.52	375.1 > 256.0 (23; 22)	375.1 > 120 (41; 10)	61
Cyproconazole	4.74-5.00	292.1 > 70.1 (23; 8)	292.1 > 125.0 (37; 12)	81
Cyprodinil	5.98-6.28	226.1 > 92.9 (45; 34)	226.1 > 76.9 (63; 34)	71
Desmedipham*	3.35-3.60	318.1 > 182.0 (19; 16)	318.1 > 136.0 (37; 12)	46
Diazinon	6.32-6.65	305.1 > 97.0 (49; 10)	305.1 > 169.1 (31; 16)	71
Difenoconazole	6.63-6.97	406.1 > 250.9 (35; 24)	406.1 > 337.2 (23; 24)	96
Dimethomorph	4.52-4.92	388.1 > 300.9 (29; 26)	388.1 > 165.1 (43; 14)	66
Diniconazole	6.86-7.00	326.1 > 70.0 (59; 12)	326.1 > 70.1 (61; 8)	76
Disulfoton sulfone	2.57-2.71	307.0 > 153.0 (17; 14)	307.0 > 171.0 (17; 14)	91
Diuron	3.00-3.20	233.1 > 72.0 (23; 8)	233.1 > 159.9 (35; 14)	81
Ethion	7.93-8.34	385.0 > 199.1 (15; 18)	385.0 > 171.0 (23; 18)	91
Ethiprole	4.36-4.55	397.0 > 350.9 (29; 30)	397.0 > 254.9 (47; 22)	156
Ethofumesate*	3.93-4.14	304.1 > 121.1 (29; 12)	304.1 > 161.2 (31; 12)	71
Ethoprophos	5.29-5.57	243.1 > 131.0 (27; 12)	243.1 > 96.6 (41; 10)	91
Etrinphos	5.98-6.29	293.1 > 125.0 (33; 12)	293.1 > 265;1 (21; 12)	66
Fenamiphos	5.58-5.87	304.1 > 217.1 (29; 20)	304.1 > 202;0 (45; 20)	11
Fenamiphos sulfone	1.82-1.92	336.0 > 188.0 (39; 16)	336.0 > 266;0 (27; 14)	131

Table 1. Retention time windows (RTWs) and MS/MS conditions for each compound (continuation…)

Compound	RTWs (min)	Quantification transition (CE[a]; V; CXP[b]; V)	Confirmation transition (CE[a]; V; CXP[b]; V)	DP[c] (V)
Fenamiphos sulfoxide	1.66-1.75	320.1 > 232.9 (33; 20)	320.1 > 171.1 (31; 16)	131
Fenarimol	5.07-5.34	330.9 > 268.0 (31; 24)	330.9 > 139.0 (47; 12)	101
Fenazaquin	9.60-9.75	307.2 > 57.0 (37; 10)	307.2 > 91.0 (87; 14)	66
Fenpyroximate	9.15-9.27	422.1 > 366.1 (25; 34)	422.1 > 135.0 (41; 12)	81
Fenpropimorph	10.47-11.00	304.3 > 147.1 (37; 14)	304.3 > 117.1 (73; 10)	66
Fluazifop p-butyl	7.75-8.15	384.1 > 282.0 (29; 26)	384.1 > 328.0 (23; 30)	116
Flumethrin*	10.68-11.2	527.0 > 267.0 (21; 24)	527.0 > 239.0 (31; 22)	46
Fluquinconazole	4.92-5.17	376.0 > 307.0 (33; 28)	376.0 > 349.0 (33; 28)	11
Flusilazole	5.88-6.02	316.1 > 247.0 (25; 22)	316.1 > 165.1 (37; 14)	86
Flutriafol	2.70-2.83	302.1 > 122.9 (35; 12)	302.1 > 109.0 (43; 12)	85
Fosthiazate	2.55-2.80	284.1 > 104.0 (27; 10)	284.1 > 227.9 (11; 20)	91
Furathiocarb	7.64-8.04	383.2 > 195.2 (17; 3)	383.2 > 252.2 (24; 3)	72
Hexaconazole	6.29-6.61	314.2 > 70.0 (53; 12)	314.2 > 159.2 (37; 12)	86
Hexythiazox	8.18-8.60	353.0 > 228.0 (21; 20)	353.0 > 168.1 (35; 16)	61
Imazalil	5.92-6.23	297.0 > 159.0 (29; 14)	297.0 > 200.9 (23; 14)	81
Indoxacarb	7.15-7.52	528.0 > 203.1 (59; 18)	528.0 > 150.1 (31; 14)	136
Iprovalicarb	5.14-5.41	321.2 > 119.0 (23; 3)	321.2 > 203.2 (12; 2)	61
Isoproturon	2.86-3.01	207.3 > 72.1 (23; 8)	207.3 > 165.1 (19;14)	71
Linuron	3.71-3.90	249.1 > 159.9 (25; 4)	249.1 > 182.0 (21; 4)	76
Malathion	4.48-4.72	330.9 > 127.1 (17; 12)	330.9 > 285.1 (11; 26)	111
Metalaxyl	3.05-3.21	280.2 > 220.1 (19; 20)	280.2 > 192.2 (25; 18)	66
Metazachlor	2.89-3.04	278.1 > 134.1 (29; 12)	278.1 > 210.1 (15; 18)	51
Metconazole	6.39-6.72	320.1 > 70.1 (59; 6)	320.1 > 125.1 (57; 12)	96
Methidathion	3.15-3.32	303.0 > 145.0 (13; 14)	303.0 > 85.1 (29; 8)	86
Methiocarb	3.90-4.10	226.1 > 169.1 (13; 14)	226.1 > 121.1 (25; 10)	76
Methiocarb sulfoxide	0.68-0.72	242.1 > 185.1 (19; 16)	242.1 > 122.1 (39; 12)	81
Methoxifenozide	4.90-5.04	369.1 > 149.0 (23; 14)	369.1 > 313.1 (11; 28)	71
Mevinphos	0.83-0.89	225.1 > 127.1 (21; 12)	225.1 > 193.0 (11; 16)	66
Monocrotophos	0.54-0.57	224.1 > 127.0 (23; 12)	224.1 > 98.0 (17; 12)	71
Monolinuron	2.16-2.28	215.1 > 125.9 (27; 12)	215.1 > 148.0 (19; 12)	91
Myclobutanil	4.64-4.88	289.1 > 70.1 (33; 10)	289.1 > 125.1 (39; 10)	91
Nuarimol	3.90-4.20	314.9 > 252.0 (31; 22)	314.9 > 81.1 (51; 8)	81
Omethoate	0.44-0.47	214.1 > 183.0 (15; 16)	214.1 > 125.0 (29; 12)	56
Oxamyl*	0.50-0.53	237.1 > 72.1 (25; 8)	237.1 > 90.0 (11; 10)	51
Paclobutrazol	4.48-4.72	294.0 > 70.1 (55; 6)	294.0 > 125;0 (55; 12)	81 / 51
Paraoxon-ethyl	2.75-3.00	276.0 > 220.0 (21; 20)	276.0 > 174.0 (33; 16)	81
Penconazole	5.90-6.21	284.2 > 70.1 (21; 8)	284;2 > 159.0 (41; 14)	46
Pencycuron	6.72-7;07	329.0 > 125.0 (31; 12)	329;0 > 218.0 (23; 20)	91
Pendimethalin	8.15-8.57	282.2 > 212.1 (15; 20)	282.2 > 91.0 (33; 8)	36
Phenthoate	5.80-6.10	321.0 > 79.1 (51; 16)	321.0 > 163.1 (17; 16)	96
Phorate sulfoxide	2.46-2.60	276.9 > 199.0 (13; 18)	276.9 > 142.9 (27; 12)	111

Table 1. Retention time windows (RTWs) and MS/MS conditions for each compound (continuation…)

Compound	RTWs (min)	Quantification transition (CE[a]; V; CXP[b]; V)	Confirmation transition (CE[a]; V; CXP[b]; V)	DP[c] (V)
Phosphamidon	1.25-1.55	300.0 > 127.0 (27; 12)	300.0 > 226.9 (19; 20)	91
Phosmet	3.42-3.59	318.0 > 133.0 (51; 12)	318.0 > 160.0 (19; 12)	96
Picolinafen	7.71-8.10	377.2 > 238.3 (35; 14)	377.2 > 145.0 (69; 14)	91
Pirazophos	6.51-6.85	374.1 > 222.1 (29; 20)	374.1 > 194.1 (43; 20)	86 / 91
Pirimiphos-ethyl	7.85-8.26	334.2 > 198.0 (32; 18)	334.2 > 182.1 (31; 18)	61
Pirimiphos-methyl	6.63-6.97	306.1 > 164.1 (29; 14)	306.1 > 108.1 (39; 10)	51
Profenofos	7.42-7.81	372.9 > 302.9 (25; 28)	372.9 > 97.0 (35; 28)	126
Propaquizafop	8.07-8.20	444.1/446.2 > 370.9 (21; 34)	444.1/446.2 > 100.0 (23; 10)	111 / 86
Propargite*	8.56-9.00	368.1 > 231.1 (15; 20)	368.1 > 175.1 (23; 16)	41
Propham	2;61-2;74	180.1 > 138.1 (13; 14)	180.1 > 120.1 (25; 14)	61
Propoxur	1.68-1.77	210.1 > 111.0 (19; 3)	210.1 > 168.1 (11; 3)	61
Pyraclofos	6.84-6.94	361.0 > 256.9 (31; 24)	361.0 > 111.0/138.0 (81; 10/ 55; 12)	111
Pyraclostrobin	6.46-6.80	388.0 > 194.1 (17; 18)	388.0 > 163.1 (33; 14)	51
Pyridaben	9.43-9.95	365.1 > 309.1 (17; 30)	365.1 > 147.2 (31; 30)	41 / 21
Pyrifenox	7.99-8.40	294.2 > 93.1 (27; 8)	294.2 > 92.1 (83; 8)	86 / 66
Pyriftalid	3.81-3.97	319.0 > 139.0 (37; 12)	319.0 > 220.1 (33; 20)	96
Pyrimethanil	4.00-4.21	200.2 > 107.1 (33; 10)	200.2 > 80.0 (39; 8)	41
Pyriproxyfen	7.99-8.40	322.0 > 96.0 (21; 10)	322.0 > 78.1 (75; 6)	71
Pyroquilone	1.60-1.85	174.1 > 132.0 (33; 12)	174.1 > 117.0 (41; 12)	91
Quinalphos	5.73-6.03	299.1 > 163.1 (33; 14)	299.1 > 147.1 (31; 14)	61
Quinoclamine	1.40-1.65	208.1 > 105.0 (33; 10)	208.1 > 89.0 (51; 8)	106
Quizalofop-P-ethyl	7.77-7.88	373.0 > 299.0 (27; 26)	373.0 > 271.0 (35; 22)	151
Spiromesifen	8.80-8.92	371.1 > 273.0 (21; 22)	371.1 > 255.1 (31; 20)	141
Tebuconazole	5.98-6.29	308.1 > 70.1 (57; 8)	308.1 > 125.1 (53; 12)	71
Tebufempirade	7.80-8.20	334.1 > 145.1 (39; 4)	334.1 > 117.1 (67; 6)	111
Temephos	8.10-8.20	466.9 > 418.9 (25; 34)	466.9 > 125.0 (41; 12)	86
Tetraconazole	5.45-5.60	372.0/374.0 > 159.0 (39; 14)	372.0/374.0 > 161.0 (39; 14)	101 / 81
Thiacloprid	0.80-0.85	253.3 > 126.0 (29; 12)	253.3 > 186.0 (21; 12)	101
Thiobencarb	6.96-7.08	258.0/260.1 > 125.0 (23; 12)	258.0/260.1 > 127.0 (25; 14)	56
Thiodicarb	2.05-2.16	355.1 > 88.1 (27; 3)	355.1 > 108.0 (21; 3)	60
Triadimefon	4.67-4.91	2940 > 197.0 (21; 18)	2940 > 225.0 (17; 20)	66
Triadimenol	4.84-5.09	296.1/298.0 > 70.1 (31; 8)	296.1/298.0 > 70.0 (33; 8)	46
Trichlorfon	0.79-0.84	257.0 > 109.0 (23; 10)	257.0 > 221.0 (15; 20)	101
Tricyclazole	1.00-1.25	190.1 > 163.0 (31; 14)	190.1 > 136.0 (39; 12)	61
Trifloxystrobin	7.20-7.57	409.1 > 186.1 (23; 16)	409.1 > 145.1 (63; 14)	66
Triflumizole	7.12-7.48	346.0 > 278.0 (15; 26)	346.0 > 73.1 (21; 8)	51
Triforin	3.51-3.69	434.9 > 389.8 (17; 36)	434.9 > 215.1 (37; 20)	56

The precursor ion for most of the pesticides was $[M + H]^+$, except for * which were $[M + NH_4]^+$.
[a]CE - collision energy potentials; [b]CXP - collision exit potentials; [c]DP - declustering potential; V - voltage

2.3. Sample preparation

The National and Agriculture Laboratory LANAGRO-MG, Ministry of Agriculture, Livestock and Food Supply (MAPA), where this study was developed, is accredited by INMETRO (National Institute of Metrology, Quality and Technology) according to ISO 17025:2005 (ISO, 2005) for the analysis of pesticides in several foodstuffs. The methods developed at LANAGRO by means of QuEChERS were used as a starting point in this study. Pesticide free samples were used as blanks for validation experiments. Some parameters that affect QuEChERS extraction were optimized (univariate analysis), such as the amount of sample (2.5, 5 and 10 g), the amount of water for sample dilution (8.5 and 10 mL), the type of extraction solvent (acetonitrile and acetonitrile:ethyl acetate, 70:30 v/v) and the type of clean-up sorbents (50 mg of PSA; 50 mg of Florisil; or 50 mg of PSA together with 50 mg of Florisil) with 150 mg of $MgSO_4$ for 500 µL of extract. The extraction salts were maintained as follows: 4 g of $MgSO_4$ and 1 g of sodium acetate. Figure 1 shows the flow chart of the QuEChERS method adapted for the analysis of pesticides in honey.

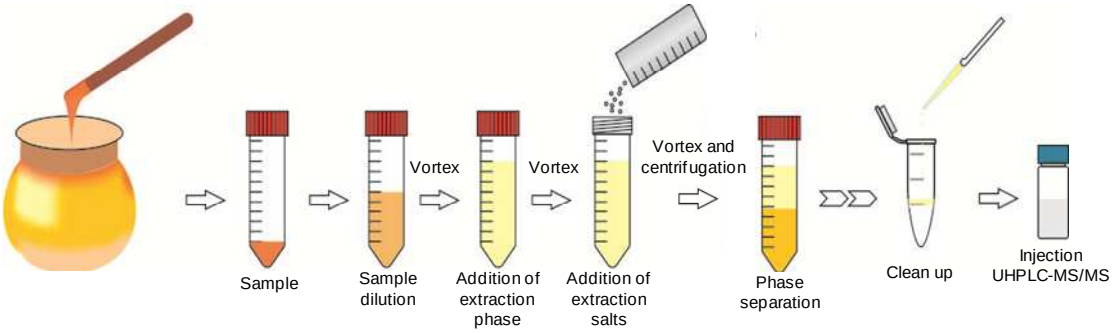

Figure 1. QuEChERS method adapted for the analysis of pesticides in honey.

2.4. Method validation

2.4.1. Selectivity and calibration curves

Validation was performed following the European Union SANCO/12571/2013 guideline (SANCO, 2013). The selectivity of the method was evaluated by injecting extracted blank samples. The absence of signal above a signal-to-noise ratio of 3 at the retention times of the target compounds was the parameter used to show that the method was free of interferences. For the preparation of analytical matrix-matched calibration curves (MMC), blank honey extracts were spiked with proper amounts of standard solutions at the final concentrations of 0.005, 0.0075, 0,010, 0.025, 0.050, 0.075, 0.100 mg/kg and injected in random order (n = 6). All solutions were prepared independently. The best type of fit for the regression curve was decided for each compound by applying the homoscedasticity test. Since the data for all analytes were heteroscedastic the weighted least squares method (WLS) was used. The fit quality and significance of the regression model employed were evaluated using the lack of fit test. The significance level used in all tests was 95%.

2.4.2. Trueness and precision

The trueness was determined on three days and three different analysts. Blank honey extracts were spiked with the analytes at four distinct levels: 0.010, 0.025, 0,050 and 0.100 mg/kg (n = 6 replicates per level). Recoveries were calculated by comparing the concentrations of the extracted compounds with those from the MMC calibration curves. These data were also used to determine the intermediate precision of the method and quantifying the measurement uncertainty (MU). Repeatability, expressed as relative standard deviation (RSD), was evaluated from replicate samples (n = 6) analyzed at the same day for each level. The intermediate precision, expressed as relative standard deviation (RSD), was evaluated through the replicates data (n = 18) of the three different days for each level.

2.4.3. Limit of detection, limit of quantification and measurement of uncertainty

The limit of detection (LOD) was experimentally determined using spiked blank honey extracts with all the pesticides. The LOD was defined as the lowest concentration of analyte that could be differentiated of the matrix signal with a signal-to-noise ratio (S/N) higher than 6. The LOQ was based on the trueness and precision data, obtained by recovery determination and was defined as the lowest validated spiked level meeting the requirements of a recovery within the range 70-120% and an RSD ≤ 20%. Measurement uncertainty (MU) was established according to ISO (International Organization for Standardization)/TS 21748:2004 (ISO, 2004) and EURACHEM guide (EURACHEM, 2000).

3. Results and discussion

3.1. Extraction method

QuEChERS was chosen for the analysis of pesticides in honey based on the description of several studies in the literature demonstrating its efficiency and good performance for extraction of pesticides in this matrix (WIEST et al., 2011; TOMASINI et al., 2012; BARGANSKA et al., 2013; KUJAWSKI et al., 2014). Another criterion used to choose the sample preparation technique was acceptable recoveries for all analytes. After investigating different conditions regarding sample weight, amount of water for sample dilution, type of extractor solvent and type of clean-up phase, the final method was established as: honey (5 g) was weighed in 50 mL tubes and spiked with proper amounts of working standard solutions of pesticides, 10 mL of water was added, and the mixture was vortexed for 30 seconds. The extraction phase, acetonitrile:ethyl acetate 70:30 with 1% acetic acid (v/v), was added and the mixture vortexed for 1 min. The extraction salts (4 g of magnesium sulfate and 1 g of sodium acetate) were added, vortexed and centrifuged at 1900 x *g* for 9 min at 20 ^{o}C. The supernatant (500 µL) was transferred to a 2 mL tube containing 150 mg of magnesium sulfate, 50 mg of Florisil and 50 mg of PSA for clean-up, and submitted to vortex and centrifugation as already described. Finally, an aliquot

of supernatant was transferred to a vial followed by injection at the UHPLC-MS/MS system. The choice of the amount of honey sample and water for dilution as well as the type of extraction solvent and clean-up salts was based on data from recovery and sample cleaning.

The original QuEChERS method consists of two steps, a salting out extraction and a dispersive SPE (dSPE) clean-up (ANASTASSIADES et al., 2003). Since in the QuEChERS approach the sample should have more than 75% of water, an initial dilution of the honey sample was required. The use of ethyl acetate associated with acetonitrile provided less colorful (yellow) extracts, making the clean-up step easier. The use of sodium acetate together with acetic acid buffered the extracts (pKa of acetic acid = 4.75) improving pesticides stability and increasing the extraction efficiency. Magnesium sulphate was used in order to ensure dryness of the sample by means of an exothermic reaction, leading to phase separation and extraction of the compounds by the acetonitrile:ethyl acetate solution. To remove the matrix interference, a clean-up step was also performed. A dispersive solid phase extraction employing PSA together with Florisil was performed. PSA had the ability to retain matrix components, such as polar organic acids, sugars and fatty acids. Florisil improved sample clean-up, due to the sugars interaction with the polar surface of this sorbent (KOESUKWIWAT et al., 2008; PRESTES et al., 2009; KUJAWSKI et al., 2014).

Figure 2 shows the flow chart of the original QuEChERS method and QuEChERS modified for the extraction of pesticides in honey.

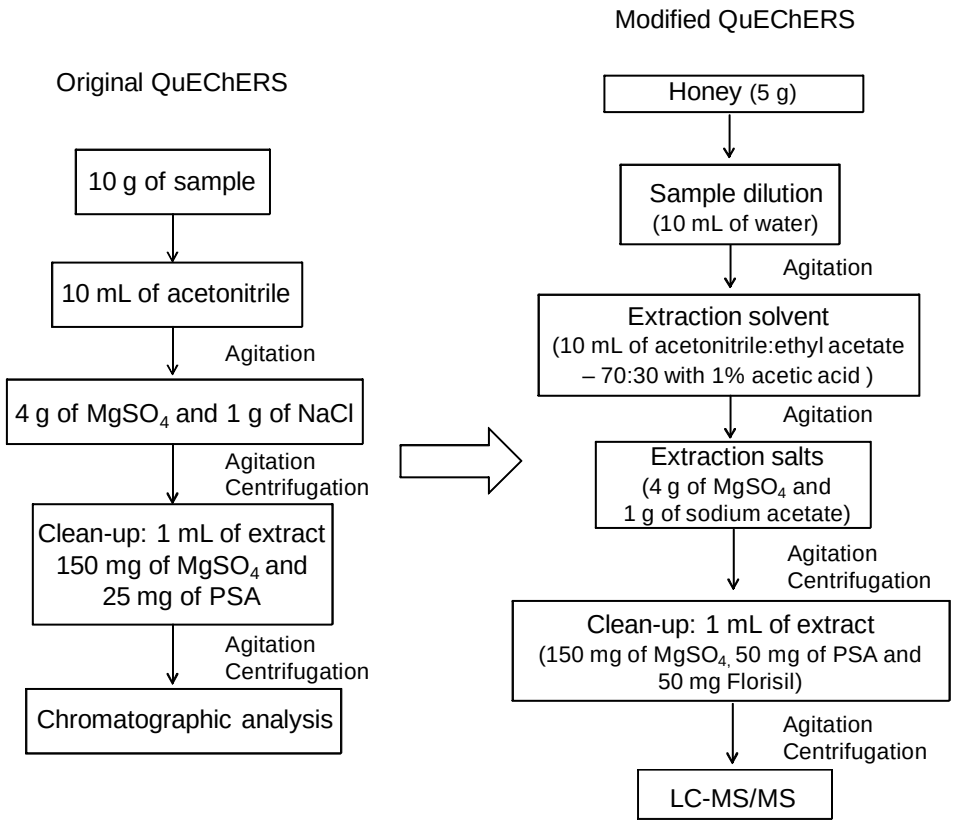

Figure 2. Steps, reagents and amounts used in the original QuEChERS method and in the QuEChERS modified for the extraction of pesticides in honey.

3.2. Method validation

According to the European Union SANCO/12571/2013 guidelines (SANCO, 2013), the precursor (parent) ion and the two transitions (quantification and identification ions) should be present with a signal-to-noise (S/N) ratio greater than 3 (in the lowest calibration level this ratio should be higher than 6); and the ratio of the quantification/confirmation transitions in the sample and the previously injected standard should not differ by more than ± 30%. Therefore, two transitions were selected for each compound (Table 1) and these criteria were evaluated. Figure 3 shows the total ion chromatogram (TIC) obtained from a blank sample spiked with all pesticides at 0.01 mg/kg. The absence of signal above a signal-to-noise ratio of 3 at the retention times of the target compounds showed that the method was free of interferences.

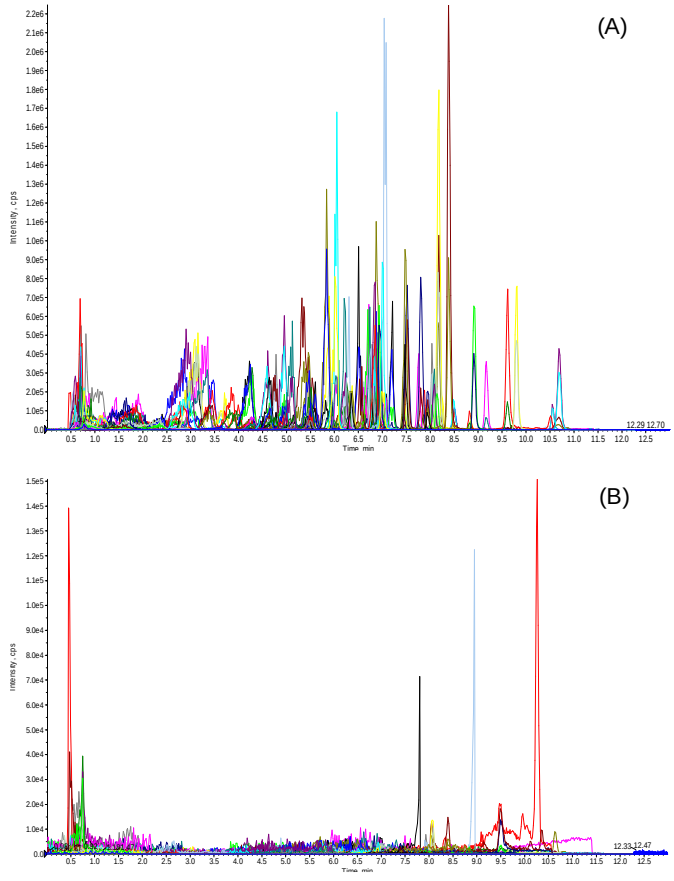

Figure 3. Total ion chromatograms (TIC) obtained by UHPLC-MS/MS (ESI positive mode) for blank honey extracts spiked with 116 pesticides at 0.1 mg/kg (A) and for a blank sample (B). The y-axis scale is different in the two chromatograms.

The criteria adopted for the selection of the analytical curve levels were the signal-to-noise ratio and the recovery results. From this evaluation five concentrations were selected: 0.010, 0.025, 0.050, 0.075, and 0.100 mg/kg. The 0.005 mg/kg concentration level was injected to confirm the LOD of the method. Over the calibration ranges selected, all the calibration curves presented significant linearity according to the lack of fit test and t-test on determination coefficients (r^2). The LOD and LOQ of the pesticides are indicated in Table 2. It can be seen that the LODs and LOQs were 0.005 mg/kg and 0.010 mg/kg, respectively, except for 3-hydroxy carbofuran, acetamipride, cyproconazole, chlorbufam, ethoprophos, fenhexamid, fentoate, fluquinconazole, penconazole, pyraclofos and propham, which had a LOQ of 0.025 mg/kg.

Table 2. Validation parameters obtained for the 116 pesticides in the developed method for honey

Compound	Average recovery (%) (Intermediate precision, % RSD)				Uncertainty measurement (%)				LOD (mg/kg)	LOQ (mg/kg)	LMR* (mg/kg)
	Concentration level (mg/kg)				Concentration level (mg/kg)						
	0.010	0.025	0.050	0.100	0.010	0.025	0.050	0.100			
3-Hydroxy carbofuran	-	95.1 (14.9)	94.1 (18.2)	97.7 (15.3)	-	18.4	13.7	13.1	0.005	0.025	-
Acetamipride	-	103.5 (17.5)	92.9 (13.5)	92.0 (17.0)	-	16.4	12.7	13.0	0.005	0.025	0.05
Alachlor	98.4 (13.4)	97.0 (13.0)	95.6 (13.5)	98.3 (10.8)	26.7	14.1	12.4	12.0	0.005	0.010	0.01
Aldicarb	94.0 (14.6)	90.5 (16.4)	99.7 (15.0)	99.3 (19.1)	24.1	14.7	12.5	13.1	0.005	0.010	0.01
Allethrin	94.5 (8.2)	99.8 (13.4)	99.2 (18.9)	95.9 (13.4)	46.4	18.5	13.9	12.9	0.005	0.010	-
Ametryn	99.8 (8.7)	94.9 (12.7)	92.7 (15.1)	100.9 (13.6)	32.5	15.2	12.8	12.5	0.005	0.010	-
Azinphos ethyl	94.2 (17.0)	103.1 (15.6)	97.7 (16.3)	86.6 (16.7)	39.4	17.5	13.7	13.8	0.005	0.010	-
Azinphos methyl	96.8 (11.4)	96.8 (13.1)	98.6 (11.2)	96.9 (12.4)	21.1	13.1	11.9	12.0	0.005	0.010	-
Azoxystrobin	94.3 (15.6)	98.2 (13.2)	98.4 (15.9)	99.7 (11.5)	34.4	15.6	12.9	12.3	0.005	0.010	0.05
Benalaxyl	102.1 (15.9)	102.3 (12.7)	98.0 (9.8)	102.9 (11.8)	24.0	13.5	11.9	12.0	0.005	0.010	-
Bitertanol	97.9 (16.0)	97.2 (16.2)	98.8 (17.0)	97.0 (13.0)	31.4	15.3	13.0	12.3	0.005	0.010	0.05
Buprofezin	102.4 (8.4)	97.5 (11.0)	94.8 (12.8)	93.1 (11.2)	29.7	14.5	12.4	12.1	0.005	0.010	0.05
Cadusafos	104.5 (19.1)	97.3 (19.5)	93.3 (19.0)	90.9 (14.9)	37.8	17.0	13.5	12.8	0.005	0.010	0.01
Carbaryl	99.5 (6.9)	97.2 (6.8)	95.5 (7.4)	93.4 (5.6)	31.7	14.6	11.9	11.7	0.005	0.010	-
Carbendazin	90.6 (10.9)	91.2 (8.7)	91.4 (11.6)	96.2 (12.8)	44.3	17.6	12.8	12.8	0.005	0.010	-
Chlorbupham	-	97.3 (17.3)	101.2 (15.3)	97.0 (13.8)	-	16.5	12.9	12.6	0.005	0.025	-
Chlorfentezine	93.0 (15.6)	93.9 (15.8)	93.8 (15.3)	82.4 (13.4)	41.3	17.4	13.1	12.7	0.005	0.010	-
Chlorpyrifos-methyl	94.1 (17.6)	88.1 (17.4)	89.2 (15.6)	90.7 (12.5)	32.9	15.8	12.8	12.3	0.005	0.010	-
Chlortiophos	94.1 (10.9)	99.1 (9.0)	97.8 (15.3)	94.5 (11.7)	34.3	15.3	12.9	12.3	0.005	0.010	-
Cinidon-ethyl	90.7 (15.2)	86.5 (15.5)	89.6 (15.4)	87.6 (16.6)	43.7	18.0	13.3	13.2	0.005	0.010	-
Cyazofamid	96.4 (13.9)	95.1 (13.7)	92.2 (13.9)	99.1 (12.9)	26.4	14.1	12.4	12.2	0.005	0.010	0.05
Cyhalofop butyl	92.9 (17.3)	93.6 (12.8)	97.4 (15.6)	91.0 (13.9)	48.2	20.0	13.0	16.1	0.005	0.010	0.05
Cyproconazole	-	90.5 (14.3)	87.7 (13.6)	97.6 (9.7)	-	18.7	13.2	12.6	0.005	0.025	0.05
Cyprodinil	100.6 (8.3)	91.7 (14.8)	102.3 (7.0)	98.1 (13.4)	25.6	14.1	11.7	12.2	0.005	0.010	0.05
Desmedipham	99.1 (11.3)	94.5 (13.4)	93.0 (13.5)	88.9 (14.7)	35.1	15.8	12.7	12.7	0.005	0.010	-
Diazinon	102.9 (10.2)	95.0 (12.8)	96.8 (12.3)	96.0 (12.6)	31.8	15.1	12.4	12.3	0.005	0.010	0.01
Difenoconazole	97.2 (13.6)	88.7 (13.8)	96.4 (13.1)	94.1 (9.5)	35.2	15.9	12.6	12.1	0.005	0.010	0.05

Table 2. Validation parameters obtained for the 116 pesticides in the developed method for honey (continuation…)

Compound	Average recovery (%) (Intermediate precision, % RSD)				Uncertainty measurement (%)				LOD (mg/kg)	LOQ (mg/kg)	LMR* (mg/kg)
	Concentration level (mg/kg)				Concentration level (mg/kg)						
	0.010	0.025	0.050	0.100	0.010	0.025	0.050	0.100			
Dimethomorph	99.2 (11.5)	96.8 (15.7)	97.7 (12.6)	99.5 (10.7)	37.5	16.4	12.6	12.3	0.005	0.010	0.05
Diniconazole	98.3 (10.7)	102.2 (10.1)	97.2 (12.4)	95.7 (12.4)	24.4	13.4	12.2	12.1	0.005	0.010	0.05
Disulfoton sulfone	99.2 (9.1)	99.1 (9.7)	99.1 (10.5)	94.9 (9.0)	18.2	12.4	11.8	11.6	0.005	0.010	0.01
Diuron	96.4 (7.2)	97.8 (6.4)	94.9 (11.9)	98.7 (9.9)	23.6	13.0	12.1	11.8	0.005	0.010	0.05
Ethion	100.5 (10.6)	94.1 (14.1)	95.2 (16.9)	92.2 (16.4)	38.3	16.6	13.2	13.0	0.005	0.010	0.01
Ethiprole	98.7 (16.5)	91.6 (17.3)	92.2 (19.2)	85.3 (18.7)	47.1	19.0	14.0	13.7	0.005	0.010	-
Ethofumesate	93.9 (9.7)	94.7 (14.3)	96.5 (13.2)	92.5 (11.7)	35.8	16.1	12.6	12.3	0.005	0.010	0.1
Ethoprophos	-	99.2 (13.4)	103.6 (14.2)	108.9 (14.9)	-	22.5	14.2	13.9	0.005	0.025	-
Etrinphos	99.9 (14.9)	100.3 (11.9)	99.5 (13.1)	91.4 (13.4)	23.3	13.4	12.2	12.2	0.005	0.010	-
Fenamiphos	87.2 (11.0)	92.9 (15.7)	82.5 (17.0)	82.0 (15.3)	44.9	18.6	13.8	13.6	0.005	0.010	0.01
Fenamiphos sulfone	97.9 (10.0)	91.9 (12.1)	90.4 (12.5)	88.2 (13.4)	35.8	15.9	12.6	12.5	0.005	0.010	0.01
Fenamiphos sulfoxide	88.8 (7.7)	87.9 (10.4)	90.6 (10.1)	85.5 (6.9)	40.2	17.3	12.8	12.2	0.005	0.010	0.01
Fenarimol	102.7 (16.9)	91.8 (13.5)	86.6 (19.6)	87.4 (18.6)	46.6	18.8	14.3	14.0	0.005	0.010	0.05
Fenazaquin	95.5 (14.2)	99.2 (14.5)	97.6 (14.0)	94.7 (11.9)	44.1	18.5	13.8	13.9	0.005	0.010	0.01
Fenhexamid	-	89.4 (16.1)	85.7 (16.1)	88.0 (15.2)	-	18.5	13.9	13.4	0.005	0.025	0.05
Fenpyroximate	96.6 (8.1)	98.7 (12.7)	98.4 (13.8)	98.1 (12.3)	22.2	13.3	12.3	12.0	0.005	0.010	0.05
Fenpropimorph	91.1 (8.4)	87.1 (12.3)	85.7 (13.1)	86.3 (8.3)	25.5	13.8	12.3	11.7	0.005	0.010	-
Fluazifop p-butyl	98.5 (9.0)	97.6 (13.0)	98.3 (8.7)	97.9 (7.5)	25.8	13.9	11.8	11.7	0.005	0.010	0.05
Flumethrin	101.1 (12.8)	97.0 (10.5)	97.7 (11.9)	92.3 (12.4)	26.3	13.8	12.2	12.1	0.005	0.010	-
Fluquinconazole	-	94.8 (17.6)	93.4 (16.5)	94.9 (15.0)	-	19.6	13.7	13.2	0.005	0.025	0.02
Flusilazole	101.8 (12.3)	98.2 (15.2)	100.3 (13.2)	95.5 (12.0)	27.9	14.6	12.4	12.1	0.005	0.010	0.05
Flutriafol	94.5 (11.0)	90.9 (7.9)	97.3 (6.0)	97.3 (8.5)	17.9	12.2	11.4	11.6	0.005	0.010	0.05
Fosthiazate	99.8 (12.5)	95.8 (12.7)	97.0 (14.0)	89.6 (8.4)	28.5	14.4	12.5	11.8	0.005	0.010	-
Furathiocarb	97.9 (11.4)	98.9 (14.9)	101.0 (14.7)	100.3 (14.2)	29.2	14.8	12.6	12.4	0.005	0.010	0.01
Hexaconazole	98.6 (15.3)	95.0 (14.5)	92.3 (14.1)	89.8 (8.4)	48.5	19.1	13.3	13.2	0.005	0.010	-
Hexythiazox	98.2 (9.4)	102.0 (14.3)	100.0 (14.6)	96.1 (8.5)	29.7	14.8	12.6	11.8	0.005	0.010	0.02

Table 2. Validation parameters obtained for the 116 pesticides in the developed method for honey (continuation…)

Compound	Average recovery (%) (Intermediate precision, % RSD)				Uncertainty measurement (%)				LOD (mg/kg)	LOQ (mg/kg)	LMR* (mg/kg)
	Concentration level (mg/kg)				Concentration level (mg/kg)						
	0.010	0.025	0.050	0.100	0.010	0.025	0.050	0.100			
Imazalil	90.8 (15.0)	93.0 (15.1)	91.0 (14.2)	92.4 (15.6)	39.9	17.1	12.9	13.0	0.005	0.010	0.05
Indoxacarb	103.6 (13.4)	101.9 (13.7)	95.7 (11.4)	101.1 (11.0)	41.8	17.4	12.7	12.5	0.005	0.010	0.05
Iprovalicarb	102.7 (12.6)	105.4 (13.6)	96.1 (14.7)	95.9 (12.3)	40.0	16.9	13.0	12.5	0.005	0.010	0.05
Isoproturon	96.7 (8.9)	99.5 (14.5)	95.1 (13.9)	95.6 (13.0)	34.1	15.7	12.7	12.4	0.005	0.010	0.05
Linuron	98.0 (11.2)	96.9 (11.4)	99.2 (13.2)	100.4 (13.0)	36.8	16.0	12.7	12.5	0.005	0.010	-
Malathion	100.5 (16.2)	103.3 (12.3)	100.7 (14.2)	103.2 (9.4)	37.0	16.1	12.8	12.1	0.005	0.010	0.02
Metalaxyl	97.5 (11.2)	101.8 (10.9)	96.5 (10.5)	98.0 (9.6)	29.7	14.5	12.1	11.9	0.005	0.010	0.05
Metazachlor	100.4 (10.3)	102.1 (12.9)	100.0 (13.8)	95.1 (11.5)	29.2	14.6	12.5	12.1	0.005	0.010	0.05
Metconazole	101.9 (11.7)	103.4 (13.3)	92.7 (17.3)	93.3 (15.3)	37.0	16.3	13.2	12.8	0.005	0.010	0.05
Methidathion	95.6 (14.0)	93.4 (13.4)	93.2 (14.0)	94.0 (17.1)	27.6	14.3	12.5	12.8	0.005	0.010	0.02
Methiocarb	103.6 (13.8)	95.5 (13.4)	97.5 (14.9)	92.7 (14.4)	40.1	17.0	13.0	12.8	0.005	0.010	0.05
Methiocarb sulfoxide	87.9 (15.8)	89.0 (10.6)	89.1 (14.5)	95.4 (10.5)	44.3	17.7	13.2	12.5	0.005	0.010	0.05
Methoxifenozide	94.1 (19.5)	101.2 (15.4)	101.4 (17.9)	93.6 (16.2)	46.8	19.3	14.1	13.2	0.005	0.010	0.05
Mevinphos	94.7 (13.9)	99.3 (11.3)	95.9 (14.3)	94.1 (14.8)	36.6	16.0	12.8	12.7	0.005	0.010	-
Monocrotophos	88.6 (18.3)	94.7 (15.8)	88.5 (14.7)	87.0 (14.6)	49.9	19.5	13.5	13.2	0.005	0.010	-
Monolinuron	100.6 (9.9)	99.9 (12.4)	95.9 (13.9)	95.3 (10.4)	33.7	15.5	12.7	12.1	0.005	0.010	-
Myclobutanil	100.9 (11.7)	102.1 (12.9)	100.4 (12.8)	104.2 (10.6)	39.3	16.7	12.7	12.3	0.005	0.010	-
Nuarimol	96.8 (12.5)	102.4 (12.5)	100.5 (13.5)	99.1 (11.9)	33.2	15.3	12.6	12.3	0.005	0.010	-
Omethoate	86.6 (15.3)	82.9 (14.2)	89.3 (17.7)	81.6 (14.4)	45.2	18.4	14.0	14.3	0.005	0.010	-
Oxamyl	100.8 (10.8)	98.0 (12.8)	98.4 (15.4)	89.7 (14.8)	49.1	19.6	14.3	15.7	0.005	0.010	0.05
Paclobutrazol	93.5 (9.8)	99.8 (12.6)	99.8 (11.0)	97.8 (12.8)	42.9	17.6	12.7	12.7	0.005	0.010	-
Paraoxon-ethyl	101.0 (8.8)	100.4 (12.0)	98.7 (11.2)	97.5 (11.7)	29.1	14.5	12.2	12.1	0.005	0.010	-
Penconazole	-	100.4 (14.4)	98.5 (12.7)	94.6 (11.6)	-	18.0	12.9	12.6	0.005	0.025	-
Pencycuron	101.2 (16.2)	98.2 (15.9)	96.3 (11.9)	96.9 (11.2)	44.4	18.2	12.9	12.6	0.005	0.010	-
Pendimethalin	100.0 (13.2)	96.1 (13.8)	91.5 (14.8)	91.7 (10.2)	29.4	14.7	12.6	12.0	0.005	0.010	0.05
Phenthoate	-	103.6 (16.3)	96.1 (16.8)	96.2 (13.3)	-	15.0	12.9	12.3	0.005	0.025	-
Phorate sulfoxide	95.3 (12.3)	98.9 (11.0)	100.1 (12.4)	95.0 (9.3)	20.1	12.8	12.0	11.7	0.005	0.010	0.01

Table 2. Validation parameters obtained for the 116 pesticides in the developed method for honey (continuation…)

Compound	Average recovery (%) (Intermediate precision, % RSD)				Uncertainty measurement (%)				LOD (mg/kg)	LOQ (mg/kg)	LMR* (mg/kg)
	Concentration level (mg/kg)				Concentration level (mg/kg)						
	0.010	0.025	0.050	0.100	0.010	0.025	0.050	0.100			
Phosphamidon	88.6 (13.1)	95.6 (13.6)	95.8 (14.3)	101.7 (9.5)	32.0	15.2	12.6	12.0	0.005	0.010	-
Phosmet	102.8 (11.6)	89.1 (11.6)	91.4 (14.1)	87.1 (14.4)	34.9	15.6	12.7	12.6	0.005	0.010	0.05
Picolinafen	102.6 (10.7)	95.2 (11.9)	90.7 (12.5)	86.7 (11.2)	39.6	16.7	12.7	12.4	0.005	0.010	-
Pirazophos	102.1 (16.8)	104.4 (15.9)	95.7 (11.4)	94.0 (15.3)	47.7	19.5	13.6	14.3	0.005	0.010	-
Pirimiphos-ethyl	96.4 (9.8)	101.6 (12.4)	98.2 (11.3)	97.5 (7.7)	30.3	14.8	12.2	11.8	0.005	0.010	-
Pirimiphos-methyl	100.8 (15.4)	101.6 (11.1)	98.6 (15.5)	96.7 (14.1)	33.0	15.2	12.8	12.5	0.005	0.010	-
Profenofos	99.4 (10.7)	99.1 (11.7)	98.7 (15.0)	100.1 (10.8)	38.8	16.5	13.0	12.3	0.005	0.010	0.01
Propaquizafop	94.7 (12.1)	98.6 (13.4)	97.0 (11.4)	96.1 (12.0)	39.3	16.8	12.6	12.5	0.005	0.010	0.05
Propargite	98.9 (10.0)	100.0 (11.3)	99.5 (13.6)	97.7 (12.4)	30.4	14.7	12.5	12.3	0.005	0.010	-
Propham	-	93.6 (16.4)	96.9 (13.5)	105.3 (14.5)	-	16.8	12.9	12.7	0.005	0.025	0.05
Propoxur	99.0 (9.2)	96.2 (12.9)	98.1 (14.7)	94.5 (15.1)	39.0	16.7	13.0	12.8	0.005	0.010	-
Pyraclofos	-	100.6 (15.8)	98.7 (14.7)	91.2 (13.7)	-	22.0	14.0	13.5	0.005	0.025	-
Pyraclostrobin	103.2 (15.8)	94.4 (16.0)	89.8 (17.2)	82.9 (18.8)	39.0	17.3	13.8	14.8	0.005	0.010	0.05
Pyridaben	98.6 (9.4)	99.3 (12.0)	98.0 (13.4)	98.3 (11.9)	27.2	14.1	12.4	12.1	0.005	0.010	0.02
Pyrifenox	96.8 (11.9)	103.7 (10.1)	98.6 (12.4)	101.0 (11.2)	34.9	15.5	12.5	12.3	0.005	0.010	-
Pyriftalid	97.2 (13.4)	99.0 (13.1)	99.6 (13.6)	101.4 (11.2)	24.1	13.6	12.3	12.0	0.005	0.010	-
Pyrimethanil	97.1 (7.7)	99.6 (11.7)	96.4 (13.5)	101.0 (8.6)	25.0	13.7	12.3	11.7	0.005	0.010	0.05
Pyriproxyfen	100.0 (9.2)	99.2 (14.2)	91.6 (17.4)	91.3 (15.8)	32.3	15.3	13.1	12.7	0.005	0.010	0.05
Pyroquilone	103.5 (9.1)	95.7 (14.0)	89.6 (13.8)	89.1 (14.4)	34.5	15.8	12.7	12.6	0.005	0.010	-
Quinalphos	101.6 (18.4)	103.0 (14.5)	95.1 (13.5)	91.9 (16.1)	31.6	15.2	12.5	12.8	0.005	0.010	-
Quinoclamine	99.9 (17.4)	101.8 (16.1)	99.6 (14.2)	90.5 (12.8)	30.2	15.0	12.5	12.3	0.005	0.010	0.05
Quizalofop-P-ethyl	103.4 (10.0)	96.4 (11.8)	91.3 (11.8)	85.5 (9.5)	37.9	16.3	12.6	12.2	0.005	0.010	0.05
Spiromesifen	96.7 (8.2)	94.5 (15.3)	90.2 (17.8)	86.9 (16.5)	36.0	16.2	13.3	13.0	0.005	0.010	0.01
Tebuconazole	96.6 (17.9)	103.4 (14.3)	94.7 (14.3)	97.7 (12.6)	44.1	14.2	13.1	12.7	0.005	0.010	0.05
Tebufempirade	99.8 (12.8)	100.4 (12.0)	97.3 (13.7)	98.7 (8.8)	35.5	15.8	12.7	12.0	0.005	0.010	0.05
Temephos	102.8 (12.1)	100.1 12.4	96.1 (13.7)	92.9 (11.8)	32.2	15.1	12.6	12.2	0.005	0.010	-
Tetraconazole	94.4 (16.2)	97.0 (14.8)	97.8 (13.5)	99.5 (13.9)	37.9	16.5	12.8	12.6	0.005	0.010	0.02

Table 2. Validation parameters obtained for the 116 pesticides in the developed method for honey (continuation…)

Compound	Average recovery (%) (Intermediate precision, % RSD)				Uncertainty measurement (%)				LOD (mg/kg)	LOQ (mg/kg)	LMR* (mg/kg)
	Concentration level (mg/kg)				Concentration level (mg/kg)						
	0.010	0.025	0.050	0.100	0.010	0.025	0.050	0.100			
Thiacloprid	98.6 (12.1)	102.0 (11.2)	96.2 (10.6)	96.1 (9.6)	31.3	11.2	12.2	12.0	0.005	0.010	0.2
Thiobencarb	94.8 (11.4)	101.8 (11.4)	97.5 (13.7)	97.2 (10.6)	45.8	18.2	13.2	12.6	0.005	0.010	0.05
Thiodicarb	95.7 (13.0)	99.6 (9.3)	95.2 (10.4)	93.7 (9.4)	24.4	13.3	11.9	11.8	0.005	0.010	-
Triadimefon	107.2 (13.9)	101.2 (13.2)	97.6 (18.3)	95.5 (15.9)	44.5	18.0	13.7	13.2	0.005	0.010	0.1
Triadimenol	100.6 (8.7)	100.7 (11.9)	96.9 (10.5)	99.0 (10.4)	36.7	16.1	12.4	12.2	0.005	0.010	0.1
Trichlorfon	94.8 (15.7)	94.7 (14.8)	89.4 (14.9)	85.1 (15.0)	37.9	16.9	13.5	15.1	0.005	0.010	0.01
Tricyclazole	96.9 (8.7)	96.5 (10.5)	94.6 (12.1)	88.0 (9.8)	20.4	12.8	12.0	11.7	0.005	0.010	-
Trifloxystrobin	105.0 (12.7)	99.6 (12.5)	97.2 (14.8)	92.7 (14.1)	27.7	14.2	12.6	12.4	0.005	0.010	0.05
Triflumizole	101.0 (17.6)	99.3 (17.5)	89.9 (17.0)	89.2 (18.1)	42.3	18.0	13.5	13.4	0.005	0.010	-
Triforin	100.1 (12.4)	99.3 (13.5)	99.2 (13.1)	94.9 (10.3)	21.3	13.2	12.2	11.8	0.005	0.010	0.01

*European Community legislation (European Union, 2015).
Weighted least squares method was the fit regression type for all analytes.

The trueness was evaluated by means of percent recoveries of honey blank samples spiked with 0.010, 0.025, 0.050 and 0.100 mg/kg of the pesticides (n = 6 replicates per level), since reference material was not available. Trueness and precision (repeatability), measured as % RSD, can be seen in Table 2. Almost all results showed recoveries in the range considered acceptable (70-120% - SANCO, 2013) as indicated in Figure 3. Some analytes (11 from 116) had recoveries out the acceptable range at the level of 0.010 mg/kg and, therefore, the LOQ was higher for these pesticides. Most of the analytes showed recoveries between 91 and 100% and the variation coefficient was, in general, within the range of 10-15% (Figure 4).

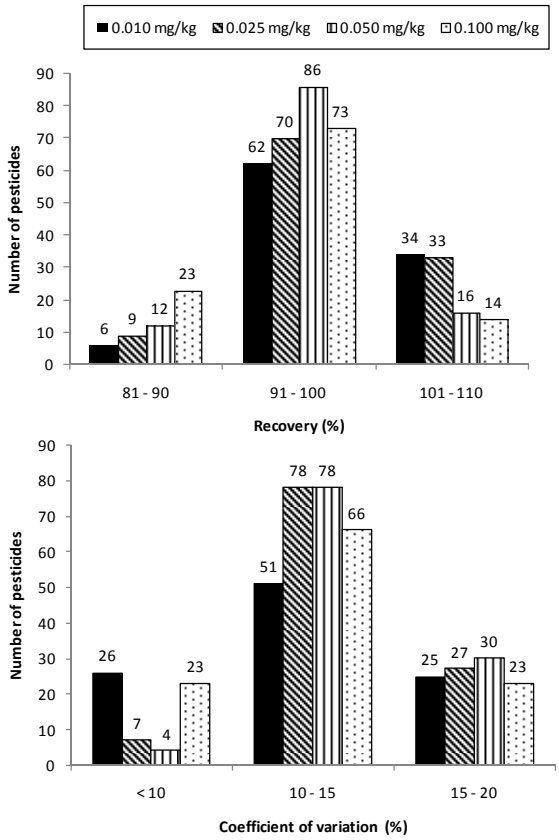

Figure 4. Recovery and coefficients of variation range of the 116 pesticides in honey at each spiked concentration evaluated.

The measurement uncertainty was based on a combination of “top-down” and “bottom-up” methodologies described in the Eurachem guide (EURACHEM, 2000). The mass measurements of the standards for the preparation of solutions, the dilution of the standard solutions, the measurements of volume of the extraction solution, the MMC curves and the intermediate precision were the main uncertainty sources associated with the method. It is known that the primary source of total uncertainty for all pesticides validated comes from the MMC curves that encompass all steps from the weighing of standards for preparation of solutions until the final quantification step, including the whole extraction process, the instrumental analysis and data statistical processing (MADUREIRA et al., 2012; CARNEIRO et al., 2013). The expanded uncertainty, expressed as percentage (MU%, Table 2), for each pesticide was determined in each fortification level for which the assessment of repeatability and reproducibility have been performed. As can be seen in Table 2, the MU

calculated for each pesticide showed values below 50%. The uncertainty values at all levels studied were in the range of 11.2%-48.5%. These results were in accordance with the acceptable criteria established in SANCO/12571/2013 document (SANCO, 2013).

3.3. Sample analysis

The optimized and validated method was applied in the analysis of 100 samples of honey of different brands. The retention time of each analyte and the relative intensities of the quantification and confirmation product ions (obtained by means of single reaction monitoring) in the real samples were compared to those of spiked blank samples at 0.010 and 0,100 mg/kg. Among the samples analyzed one of the 66 samples of Minas Gerais presented trichlorfon at 0.029 mg/kg. This result is above the maximum residue level (MRL) established by the European Union (0.01 mg/kg). Trichlorfon (dimethyl (2,2,2-trichloro-1-hydroxyethyl) phosphonate) is an organophosphate (OP) insecticide used to control a variety of pests and domestic animal ectoparasites and endoparasites (ERASLAN et al., 2010). Probably, this contamination has occurred due to the manipulation of this pesticide near the hives with the aim to control parasites in domestic animals or livestock. The presence of this insecticide in honey is worrisome for susceptible populations, including pregnant women and children (WHYATT et al., 2004). According to epidemiological investigations the fetal exposure to OP pesticides can cause inhibition in fetal growth and shortening of the period of gestation (ESKENAZI et al., 2004).

3.4. Participation in proficiency tests

The validated method was applied in the analysis of honey in a proficiency test. The received sample was submitted to analysis to identify and quantify all possible pesticides within the scope of the laboratory. To analyze the sample, a matrix-matched calibration curve was prepared with a blank extract. No false negative and no false positive results were reported and the z-scores for the

identified analytes (from -1.54 to 0.89) demonstrated the method suitability fitness for the purpose, concerning the acceptable limit of ± 2.0. The identified analytes were carbendazim, chlorpyrifos methyl, flumethrin, malation, mevinphos, thiaclopride, cypermethrin, deltamethrin and boscalid. This method will be used in routine analysis of official samples of honey from the Brazilian pesticide residues monitoring program.

4. Conclusions

The validated method using a modified QuEChERS technique as sample preparation and UHPLC-MS/MS was suitable for multiresidue detection and quantitation of 116 pesticides in honey samples. Recoveries between 81.6 to 108.9%, coefficient of variation lower or equal to 20% and expanded uncertainty of up to 48.5% were obtained. The limits of detection (LOD) were 0.005 mg/kg and limits of quantification (LOQ) were 0.01 and 0.025 mg/kg. Accuracy and precision (in intermediate precision conditions) satisfied the European Community recommendations for pesticide residues in SANCO N^{o} 12571/2013 document. In a general way the samples of honey showed appropriate quality in terms of pesticide residues. The validated method showed to be fast, efficient and reliable and can be used in the monitoring of pesticides in honey and attend the Brazilian National Plan for Residues and Contaminants (PNCRC).

CAPÍTULO III - SYNEPHRINE AS POTENTIAL BIOMARKER FOR DETERMINATION OF ORANGE HONEY AUTHENTICITY

Abstract

Monofloral honeys, coming from the nectar of one plant species such as orange honey are increasingly sought by consumers. Botanical classifications of honey are traditionally performed by melissopalynological analysis. This analytical technique is based on recognizing pollen types found in honey samples, however, it presents some disadvantages, including time consuming and dependency on the qualification and judgment of the analyst. This study evaluates the use of synephrine as a potencial biomarker for determination of orange honey authenticity. A HPLC-MS/MS method was optimized and the final method was established as: extraction with 5% trichloroacetic acid and a clean up step with Florisil 30 mg. It was validated according to the European Union guidelines and presented limits of detection and quantification of 0.66 ng/g and 1 ng/g, respectively, and mean recoveries of 83.7% ± 6.6% and 7.9% coefficient of variation. The total chromatographic run time was 8 min. Synephrine was detected in honeys with some contribution of citrus flowering; and not detected in honeys without contributions from citrus flowers. It was found in citrus fruits and flowers but not in others apiculture flowers. This amine can be used as an indication of presence of citrus species in honey samples.

Keywords: synephrine; orange honey; citrus honey; honey; amines; botanical origin; biomarker

1. Introduction

Honey is a natural food known for its nutritional and medicinal value. Honey is often used as a sugar substitute due to its sweetness, desirable color and flavor characteristics. Also, it is employed as ingredient or natural preservative in many foods, such as fruits and grains (SILVA et al., 2008; PYRZYNSKA & BIESAGA, 2009). Chemically, it is composed of a mixture of sugars, with monosaccharides representing about 75% of the sugars found in honey, along with 10-15% disaccharides and small amounts of other sugars (SILVA et al., 2016). However, the percentage of sugars varies depending on the raw material used for its production (QUEIROZ et al., 2007). Other components are also present in minor proportions, such as minerals (calcium, copper, iron, magnesium, phosphorus, potassium), proteins, amino acids, vitamins, flavonoids, pigments, and several organic acids with antioxidant properties including chrysin, pinobanksin, vitamin C, catalase and pinocembrine (FALLICO et al., 2004; DOWNEY et al., 2005; FINOLA et al., 2007; SILVA et al., 2008; BLASCO et al., 2011; SILVA et al., 2016).

The quality of honey can be affected by several factors including types of bees, presence of sucking insects, its botanical origin, geographical location, climate conditions, ripening stage, as well as the processing and storage conditions. For this reason, they may show different consistency, color, flavor and aroma (KOMATSU et al., 2002; SILVA et al., 2004; DOWNEY et al., 2005; SILVA et al., 2008; SILVA et al., 2016). Furthermore, the consistency and texture are also influenced by the content and type of protein present, which provides gelatinous characteristics for some honeys and reduces surface tension, making it more likely to retain air bubbles and to foam (QUEIROZ et al., 2007).

The color pure honey may vary from yellow to reddish yellow, with its own smell, sweet and distinctive flavor as well as liquid aspect, density, viscosity and translucence (SILVA et al., 2004). The color of honey, which is related to the flavor, depends on its age and the nectar source. The determination of color is a useful classification criterion for monofloral honeys. Darker colored honey have stronger flavor whereas lighter colored types have mild flavor, which are

preferred by consumers, and therefore have the highest prices (BOFFO et al., 2012).

The most common types of plants used for honey production are eucalyptus, citrus and wild flowers (KOMATSU et al., 2002). Honey can be produced from the nectar of a single plant species - monofloral - or more than one species - polifloral (BASTOS et al., 2002). Generally, a monofloral honey presents a characteristic aroma and taste which makes it especially appreciated among the consumers (FALLICO et al., 2004; SILVA et al., 2008).

Citrus honey is considered one of the best monofloral honeys. In addition to the appreciated flavor, the floral fragrance, which is related to its content of methyl anthranilate, is exclusive of this type of honey. It is also quite popular. It is characterized by a white color, intense odor, mild flavor and fine crystallization (VIÑAS et al., 1992; KOMATSU et al., 2002; TERRAB et al., 2003; SESTA et al., 2008).

Evaluation of orange honey authenticity is important in the context of consumer protection, quality control and trade purposes (VERZERA et al., 2014). The most commonly used approach is the melissopalynological analysis, which consists in a microscopic examination to perform the botanical classification of honey by means of identification and quantification of the percentage of pollen. Although this method is widely used, it has been considered of little value for the citrus genus honey, since the pollen of these samples is considered ‘under represented’ (RODRIGUEZ et al., 2010; ESCRICHE et al., 2011; KUS & RUTH, 2015). When compared to other honeys, the amount of pollen present in citrus honey is lower, considering the strongly present characteristics of flavor and taste (RODRÍGUEZ et al., 2010; ESCRICHE et al., 2011).

Due to limitations associated with melissopalynological analysis, methods involving chemical components of citrus honey have been developed for authenticity determination. The use of phenolic compounds, flavonoids, volatile compounds, sugars and others have been suggested for the discrimination of honeys and has been used as a tool for studying the floral origin of orange honeys (SERRANO et al., 2004; LIANG et al., 2009; ESCRICHE et al., 2011; BOFFO et al., 2012; VERZERA et al., 2014). Although these methods are

important for characterization of orange honeys, they are complex and may require a long time of analysis. The proposed method using synephrine as a biomarker for identification of orange honeys is a simple method that can help characterize this important foodstuff.

Synephrine is a sympathomimetic amine, which causes vasoconstriction, increased blood pressure and relaxation of the bronchial muscle (Figure 1) (STEWART, NEWHALL & EDWARDS, 1964; KUSU et al., 1996; VIEIRA et al., 2007). Furthermore, synephrine is useful in reducing fat mass in obese humans since it stimulates lipolysis and raises metabolic rate and oxidation of fat through increased thermogenesis (TSUJITA & TAKAKU, 2007; VIEIRA et al., 2010). This amine is characteristic of citrus fruits and has been already used as biomarker for determination of authenticity in orange juice and orange soft drink (STEWART & WHEATON, 1964; VIEIRA et al., 2007). Therefore, the aim of this study was to develop and validate a method for the analysis of synephrine in orange honeys and to evaluate the use of synephrine levels as an authenticity index for these samples.

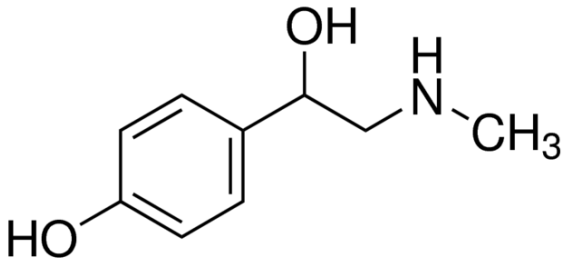

Figure 1. Chemical structure of synephrine.

2. Experimental

2.1. Honey samples

Honey samples from *Apis mellifera* bees, including citrus (*Citrus* sp., 8), vernonia (*Vernonia* sp., 2), eucalyptus (*Eucalyptus* sp., 3) and wildflower (16), were purchased from consumer stores and 'aroeira' honey (*Myracrodruon urundeuva,* 5) was provided by Serviço de Recursos Vegetais e Opoterápicos (SRVO-FUNED). Eucalyptus honey was used as blank. The samples were stored at ambient temperature (20 ^{o}C) until analysis.

2.2. Chemicals and reagents

Trichloroacetic acid (Neon, Vila Prudente, SP, Brasil), hydrochloric acid (Quimica Moderna, Barueri, SP, Brasil), glycerin (Furlab, Campinas, SP, Brasil), polymerically bonded ethylenediamine-N-propyl phase (PSA) (Agilent Technologies, Lake Forest, CA, USA) and Florisil (Sigma-Aldrich, Saint Louis, MO, USA) were of analytical grade. Synephrine and L-norvaline were both from Sigma-Aldrich (Saint Louis, MO, USA). Ultrapure water was obtained from Milli-Q Plus system (Millipore Corp., Milford, MA, USA).

2.3. Chromatography coupled to mass spectrometry

Chromatography was performed on an Agilent (Santa Clara, CA, USA) 1200 HPLC coupled to a 5500 Triple Quadrupole mass spectrometer (Applied Biosystems, MDS SCIEX, Ontario, Canada). Chromatography was carried out using a Luna C18 column (150 x 2.0 mm, 3 µm particle size) and a mobile phase consisting of water acidified with 0.1% formic acid at a flow rate of 0.2 mL/min. The injection volume was 10 µL and the column temperature was set at 20 ^{o}C. The chromatographic run was 8 min.

The mass spectrometer was operated using electrospray ionization (ESI) in the positive ion mode. Instrument settings, data acquisition and processing were controlled by Analyst software (Version 1.6, Applied Biosystems). Source

parameters were optimized as follows: ion spray voltage 5.500 kV for ESI (+), curtain gas 20 psi, collision gas 4 psi, nebulizer gas and auxiliary gas 20 psi and ion source temperature 500 ^{o}C. Retention time, precursor ion, transitions, optimal declustering potential (DP), collision energy potentials (CE) and collision exit potentials (CXP) for synephrine and norvaline are shown in Table 1.

Table 1. Retention time windows (RTWs) and MS/MS conditions for synephrine and norvaline (internal standard)

Compound	Q1 Mass	Q3 Mass	RTWs (min)	DP (volts)	CE (volts)	CXP (volts)
Synephrine	168.0	135.0	5.8 - 6.2	116	27	16
	168.0	107.0	5.8 - 6.2	116	41	10
Norvaline	118.0	72.5	5.0 - 5.5	41	25	12

DP= declustering potential; CE= collision energy potentials; CXP= collision exit potentials.

2.4. Sample preparation

The study was undertaken at Laboratorio de Bioquimica de Alimentos – LBqA, UFMG, which is accredited by INMETRO (National Institute of Metrology, Quality and Technology) according to ISO 17025:2005 (International Organization for Standardization, 2005) for the analysis of histamine in fish. Therefore, the methods developed at LBqA for analysis of amines in food were used as a starting point in this study. Individual stock solutions were prepared at 100 µg/mL and 10 µg/mL in HCl 0,1 M for synephrine and L-norvaline, respectively. Honey samples (1 g) were weighed in 50 mL tubes and spiked with proper amounts of working standard solution of synephrine and norvaline (10 ng/mL, internal standard). Afterwards, 9 mL of 5% trichloroacetic acid (TCA) was added and the obtained sample was vortex mixed (Velp Scientifica, Wizard, Usmate, Italy), sonicated (LS Logen Scientific, Diadema, São Paulo, Brazil) and centrifuged (Jouan MR23I, Saint Herblain, France). After centrifugation, the supernatant was filtered through qualitative paper (Qualy, São José dos Pinhais, Paraná, Brasil). The filtrates were collected and the volume was brought up to 10 mL in calibrated volumetric flasks. The filtrate (1

mL) was transferred to a 2 mL tube containing Florisil and polymerically bonded ethylenediamine-N-propyl phase (PSA) for clean-up, and submitted to vortex and centrifugation (Eppendorf 5424R, Hauppauge, New York, USA). Finally, the extracts were filtered through qualitative filter paper and 0.45 µm pore size membrane filter (Millipore Corp., Milford, MA, USA) prior to injection into the HPLC-MS/MS system.

2.4.1. Optimization of parameters affecting synephrine extraction from honey

Initially, a Plackett‑Burman design was used to screen the main factors that could affect recovery of synephrine from honey. The design included 12 tests and three repetitions at the central point. The variables investigated were vortexing time (X_1 = 30, 90 and 150 s), vortexing speed (X_2 = 100, 200 and 300 x *g*), relative centrifugal force (X_3 = 1000, 11000 and 21000 x *g*), centrifugation time (X_4 = 2, 6 and 10 min) and ultrasound time (X_5 = 0, 15 and 30 min).

Afterwards, based on the results obtained with the Plackett‑Burman design, a Central Composite Rotational Design (CCRD) was conducted with three replications at the central point to optimize the recoveries of synephrine. The variables considered were centrifugation time (min), ultrasound time (min) and vortexing time (s). The temperature and relative centrifugal force were set at 4 ^{o}C and 11000 x *g*, respectively, and vortexing speed at 300 x *g*. The experimental values and coded levels for the independent variables used in the CCRD are indicated in Table 2. The statistically significant experimental results from the CCRD were fitted to models for synephrine with the coded independent variables. The optimized extraction conditions that provided the best recoveries were confirmed using six replicates.

Table 2. Experimental values and coded levels of the independent variables used in the Central Composite Rotational Design during optimization of conditions for the extraction of synephrine from honey

Independent variables	Code units	Coded variable levels				
		-1,68	-1	0	+1	+1,68
Centrifugation time (min)	X_1	1	4	8	12	15
Ultrasound time (min)	X_2	1	7	15,5	24	30
Vortexing time (s)	X_3	40	60	90	120	140

2.4.2. Optimization of the variables affecting synephrine clean up

Another Plackett‑Burman design was used to screen the factors which could affect synephrine extracts clean up. The design was the same as used for the optimization of the extraction procedure and included 12 tests, six repetitions at the central point and five independent variables. The variables included were amount of sorbents (X_1 = 20, 50 and 80 mg), type of sorbents (X_2 = Florisil and; Florisil and PSA), vortexing time (X_3 = 20, 30 and 40 s), relative centrifugal force (X_4 = 1000, 9000 and 17000 x *g*) and centrifugation time (X_5 = 1, 5 and 9 min). When two sorbents were used, the same amount was used for each.

Then, a Central Composite Rotational Design (CCRD) was performed based on Plackett‑Burman design results. This experiment was conducted with three replications at the central point to optimize the recoveries of synephrine from honey. The variables were amount of sorbent (mg), centrifugation speed (*g*) and centrifugation time (min). The centrifugation temperature and vortexing speed were set at 22 ^{o}C and 10 s, respectively. The experimental values and coded levels for the independent variables used in the CCRD are presented in Table 3. The optimized extraction conditions that provided the best recoveries were confirmed using six replicates.

Table 3. Experimental values and coded levels of the independent variables used in the Central Composite Rotational Design to determine the optimum conditions of clean up of synephrine in honey

Independent variables	Code units	Coded variable levels				
		-1.68	-1	0*	+1	+1.68
Amount of sorbent (mg)	X_1	13	30	55	80	97
Centrifugation speed (g)	X_2	1000	5000	11000	17000	21000
Centrifugation time (min)	X_3	1	3	5.5	8	10

*Center point

2.5. Method validation

Validation was performed following the European Commission guidelines (EC, 2002), considering linearity, precision, accuracy, specificity, recovery, and limits of quantification and detection. For the preparation of analytical matrix-matched calibration curves (MMC), blank honey extracts were spiked with synephrine at concentrations of 1, 5, 9, 13, 17 and 21 ng/mL. Linearity was assessed by six-point calibration curves in triplicate in three consecutive days. The curves were constructed by plotting the peak area versus synephrine concentration and, by means of linear regression (Ordinary Least Square Method), the equations and the correlation coefficient were determined.

Precision and accuracy were evaluated by determining recoveries of synephrine in a set of blank samples fortified with the analyte to yield 5, 13 and 21 ng/mL. Each level was analyzed in six replicates, repeated three times at three different days with different analysts (n = 18). The concentration of synephrine in each sample, the mean concentration, the standard deviation (RSD) and the coefficient of variation (%) of the fortified samples were calculated.

The specificity of the method was verified by means of an appropriate number of blank samples (n = 20) analyzed and checked for interferences (signals, peaks, ion traces) in the region of interest where the target analyte was expected to elute.

The limit of detection was based on the noise at the retention time of synephrine of 20 independent blank samples and expressed as the analyte concentration corresponding to mean blank sample value +3s (standard deviation). The lowest point of the calibration curve was used as the quantification limit.

2.6. Application of the method

The validated method was used to determine the concentration of synephrine in *citrus*, wildflower, *eucalyptus*, *vernonia* and 'aroeira' honeys as described previously. Furthermore, the method was also applied for analysis of four apiculture flowers (*Vernonia polyanthes, Montanoa pyramidata, Tithonia diversifolia* and *Clerodendron speciosus*) widely available in Brazil. The analyses were performed in triplicate.

2.7. Pollen analysis

Qualitative melissopalynology was performed according to Belay et al. (2015), using the non-acetolytic method. Honey (10 g) was weighed into 50 mL tubes, dissolved in 20 mL of distilled water and divided into two 15 mL tubes. The solution was centrifuged for 7 min at 1800 *g* (Jouan B4i, Saint Herblain, France). The supernatant was discarded and another 5 mL of distilled water was added to completely dissolve the remaining sugar crystals and centrifuged for 7 min at 1800 *g*. The supernatant was discarded; the residue was spread evenly with a micro spatula on a microscope slide and fixed with flame. One drop of glycerin jelly was applied to the cover slip and the sample was examined through the microscope (Olympus BX50). The samples were identified using reference slides. This analysis was undertaken at Serviço de Recursos Vegetais e Opoterápicos, Fundação Ezequiel Dias (Funed).

2.8. Statistical analysis

The Plackett-Burman and CCRD experiments were performed using MINITAB® 16 (Minitab Inc., State College, PA, USA). The statistical significance was determined by analysis of variance and F test ($p \leq 0.10$).

3. Results and discussion

3.1. Optimization of the extraction method

Trichloroacetic acid has been the extracting solvent of choice for bioactive amines, because it efficiently extracts aromatic and aliphatic amines and it is also safer to work with compared to other acids, like perchloric acid, which is explosive (FERNANDES & GLORIA, 2005). The concentration of 5% was used in several studies to extract amines in food (GUIDI & GLORIA, 2012; BANDEIRA et al., 2012; PAIVA et al., 2015; EVANGELISTA et al., 2016).

The recoveries from the Plackett-Burman design ranged from 72.8 to 83.3%. Only centrifugation time affected the results ($p = 0.012$) (Figure 2a). The significance level of 0.1 was used to avoid exclusion of some important variable in the subsequent design (RIGUEIRA et al., 2011). Since centrifugation time affected significantly synephrine recovery, it was further investigated in the CCRD along with ultrasonication and vortexing times. The relative centrifugal force and vortexing speed were set at 11000 x *g* and 300 x *g*, respectively.

In the CCRD, recoveries ranged from 90.6 to 114.3% and only vortexing time significantly ($p = 0.026$) affected recoveries. The optimization tool "Response optimizer" from Minitab software was used to verify if vortexing time could improve recovery to nearly 100%. After optimization, vortexing time was set at 75 s and the following conditions were established: 1 g of sample, 9 mL 5% trichloroacetic acid, 4 min centrifugation time, 11000 x *g* relative centrifugal force, centrifugation temperature of 4 ^{o}C and the used of ultrasound was not required. The optimized conditions for the extraction of synephrine were confirmed by means of six extractions, providing average recovery of 96.8 ±

4.5%, and coefficient of variation of 4.6%. The recoveries obtained are acceptable according to European Union guidelines (EC, 2002).

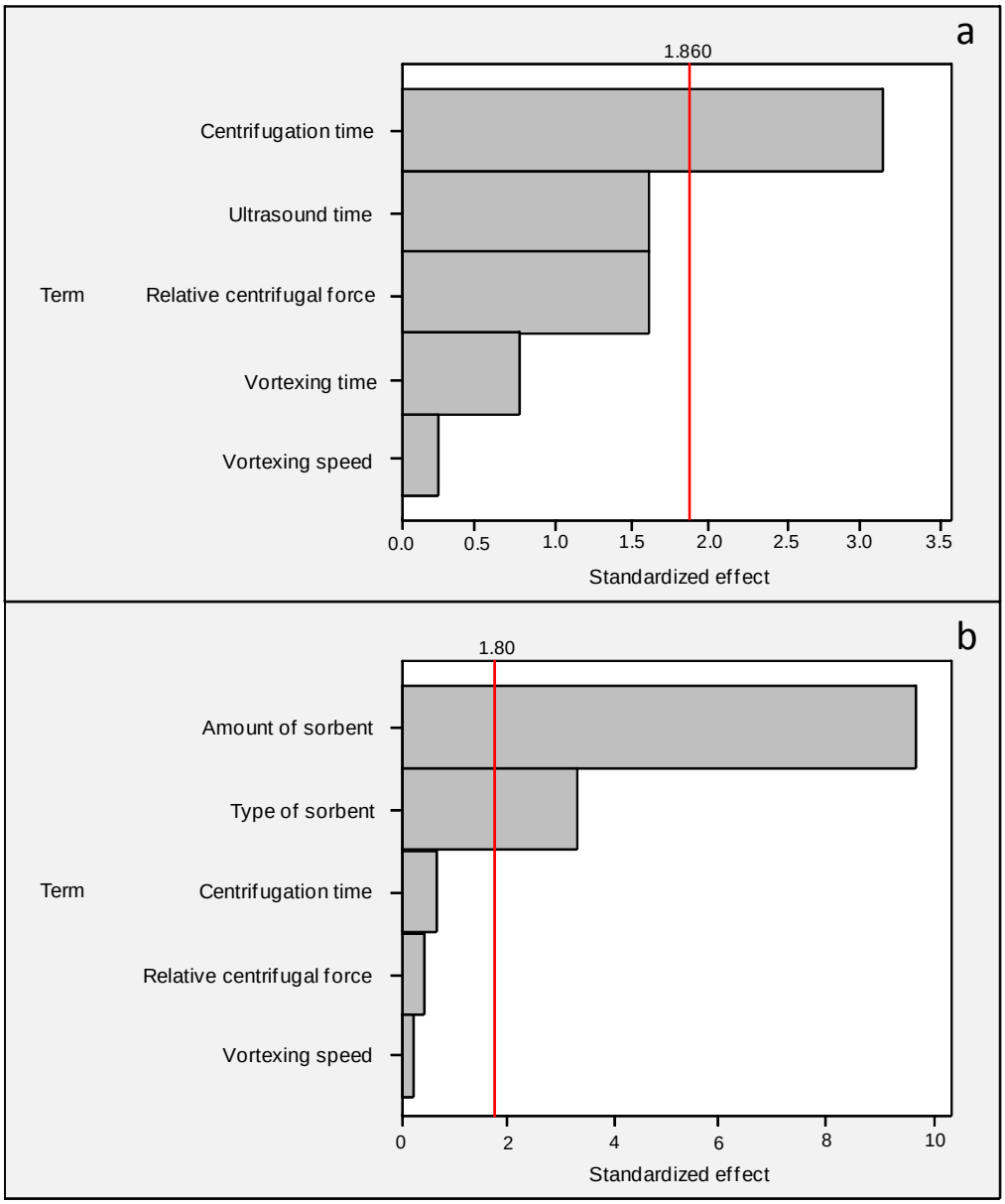

Figure 2. Pareto charts obtained in the optimization of (a) the extraction procedure and (b) the sample clean-up of by means of Plackett-Burman design for LC-MS/MS analysis of synephrine in honey.

A second Plackett-Burman design was used to optimize sample clean up by means of dispersive solid phase extraction employing PSA and Florisil. Among them, PSA has the ability to retain matrix components, such as polar organic acids, sugars and fatty acids. Whereas Florisil can improve sample clean-up, due to interaction of sugars with its polar surface (KUJAWSKI et al.,

2014; TETTE et al., 2016b). The recoveries ranged from 36.6 to 94.9 % and the results showed that the amount of sorbent (p = 0.000) and type of sorbent (p = 0.005) influenced recovery (Figure 2b). Pareto chart showed that the amount of sorbent had a more important effect compared to the type of sorbent.

Based on these results, the subsequent design was performed only with Florisil and vortexing time, relative centrifugal force and centrifugation time were set at 10 s, 17000 x *g* and 5 min, respectively. CCRD showed recoveries from 49.2 to 97.7% and the only significant variable was the amount of sorbent (p = 0.000). Higher amounts of Florisil provided lower recoveries and, according to the optimization tool "Response optimizer" from Minitab software, optimal recovery (~100%) would be achieved with the lower amount of Florisil tested (13 mg). Nevertheless, 30 mg of Florisil was chosen because this amount provided less colorful (yellow) extracts and acceptable recoveries.

After optimization, the established conditions for honey extracts clean-up for synephrine analysis were 30 mg Florisil, 10 s vortexing time, 17,000 x *g* relative centrifugal force, 5 min centrifugation time and 22 ^{o}C centrifugation temperature. These conditions provided average recovery of 83.7 ± 6.6% and coefficient of variation of 7.9%.

3.2. Method validation

The analytical matrix-matched calibration curves for synephrine were linear within the range 1-21 ng/mL, with a regression coefficient higher than 0.998 and typical standard curve: y = 16812x + 9546. The retention time of synephrine in the calibration curves constructed using solvent was different from that in matrix-matched calibration curves (MMC); therefore, MMC was used. Indeed, matrix effect in honey can result from the high levels of carbohydrates, such as glucose and fructose (TETTE et al., 2016b).

According to Table 4, the average accuracy (n = 18) determined at three different concentration levels was 79.7%. The coefficient of variation of repeatability (CV_r) ranged from 1.8% to 4.5% and the CV of reproducibility (CV_R) varied from 3.4% to 7.4%. Recoveries were within the acceptable range

(50 - 120%) established by EC (2002), which confirms the applicability of the method in the selected range.

Table 4. Precision and accuracy during synephrine determination in honey by LC-MS/MS

Nominal concentration (ng/mL)	Average concentration (ng/mL ± sd)	Precision (%)		Accuracy (%)
		CV_r	CV_R	
5	3.94 ± 0.13	4.5	3.4	78.8
13	10.63 ± 0.48	1.9	4.6	81.8
21	16.52 ± 1.23	1.8	7.4	78.7

n = 18; sd – standard deviation; CV_r – coefficient of variation of repeatability; CV_R – coefficient of variation of reproducibility.

The specificity of the method was verified by analyzing the chromatograms. Two transitions (quantification and identification ions) were selected for synephrine. The retention time of the synephrine peak was 6.0 min and no significant interference was detected at the same retention time when blank samples were analyzed (n = 20) (Figure 3). The LOD and LOQ were 0.66 ng/g and 1 ng/g, respectively.

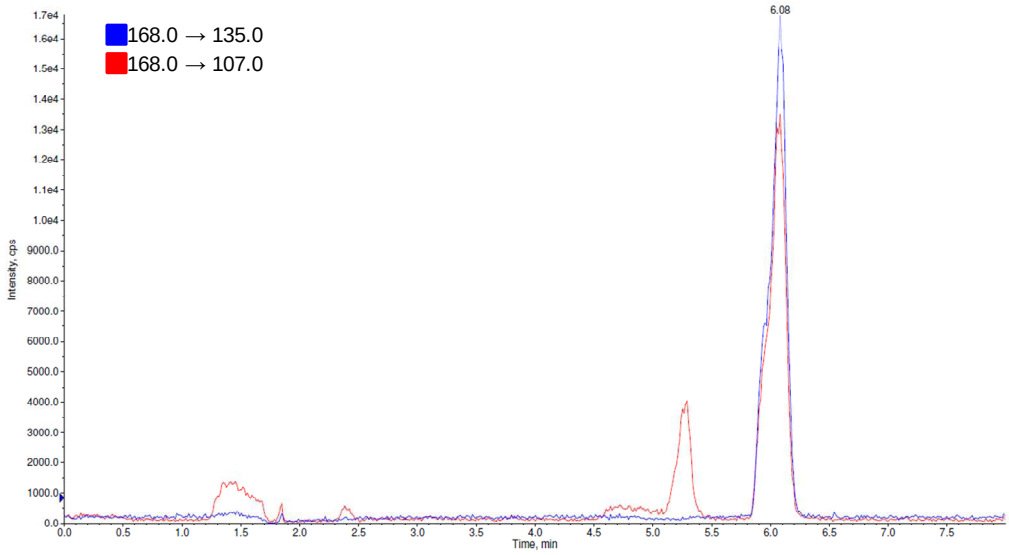

Figure 3. Total ion chromatograms (TIC) obtained by HPLC-MS/MS (ESI positive mode) for blank honey extract spiked with synephrine at 9 ng/mL.

3.3. Synephrine in honey and flowers

The optimized and validated method was used in the analysis of honey of different floral origins. Synephrine was detected in seven orange honeys (n=8), but not in other types of honey (Table 5). The concentration of synephrine in the samples ranged widely, from 79.2 to 432.2 ng/g. Synephrine is one of bioactive amine found in orange juice. It is characteristic of citrus species and it is not commonly detected in other food products (GLORIA, 2005). In fact, Vieira et al. (2007) detected synephrine in every orange sample analyzed at average concentration of 16.0 mg/mL. They successfully proposed the use of synephrine as an index of the amount of orange juice added to soft drinks.

Wildflower honey samples from two states of Brazil were also analyzed for synephrine (Table 5). Among samples from the state of Minas Gerais (n=8), synephrine was detected in one, at low concentration (22 ng/g). However, six out of 8 samples from the state of São Paulo contained synephrine at concentrations ranging from 9.4 to 236.5 ng/g. This result suggests the contribution of orange to wildflower honey from Sao Paulo. In fact, São Paulo is the main producer of orange in Brazil, concentrating the highest numbers of orange trees (BRASIL, 2016b). Many plant species can contribute with the nectar of polifloral honeys; however, geographical location of beehive plays a major role. Every day during pollinating agricultural crops, 10,000 to 25,000 honeybee workers (*Apis mellifera)* make an average of 10 journeys to explore roughly 7 km^2 in the area near their hive (BASTOS et al., 2002; RISSATO et al., 2007; SILVA et al., 2008).

To make sure that synephrine would be a reliable index of authenticity of orange honey, it is important to confirm that it is present in citrus flowers but absent in others bee flowers. Although the method has been developed for honey, it was also used in the analysis of different species of citrus flowers and also of other types, as well as of four common apiculture flowers. According to Table 6, all citrus flowers presented synephrine, the concentration ranged widely, from 0.055 to 1932,6 ng/g. The largest concentration was found in *Citrus reticulata* (Blanco).

Table 5. Synephrine levels in monofloral honeys - orange (*Citrus* sp.), *Eucalyptus* sp, *Vernonia* sp and *Myracrodruon urundeuva* honeys - and wildflower honeys from the states of Minas Gerais and São Paulo, Brazil

Honey type	Synephrine levels (ng/g)
Monofloral honeys	
Orange (*Citrus* sp.)	
A	166.3
B	nd
C	79.2
D	227.3
E	218.6
F	177.6
G	304.6
H	432.2
Eucalyptus sp.	
Three different brands (I, J & K)	nd
Vernonia sp.	
Two different brands (L & M)	nd
Myracrodruon urundeuva	
Five different brands (N, O, P, Q & R)	nd
Wildflower honeys – Minas Gerais	
S1	<LOQ
S2	nd
S3	nd
S4	nd
S5	nd
S6	22.0
S7	nd
S8	<LOQ
Wildflower honeys – São Paulo	
S9	nd
S10	43.8
S11	9.5
S12	236.5
S13	29.0
S14	9.4
S15	nd
S16	31.3

nd = not detected (LOQ = 1 ng/g).

The orange varieties *Citrus sinensis* (L.Osbeck) also presented high concentrations. This species is the main citrus species produced in Brazil, was introduced in the country in the 1500s. Brazil is today responsible for 60% of world production of orange juice and also the export champion of the product

(VIEIRA et al., 2007; BRASIL, 2016b). Synephrine not was detected in other apiculture flowers.

Table 6. Synephrine levels in citrus flowers

Samples	Synephrine levels (µg/g)
Citrus sinensis (L. Osbeck) – 'Bahia'	
A	813.0
B	523.5
Citrus sinensis (L. Osbeck) – 'Serra d'água'	
A	989.5
B	1090,1
Citrus limetta	
A	585.5
B	684.1
Citrus latifolia (Tanaka)	
A	0.057
B	0.055
Citrus reticulata (Blanco)	
A	1932,6

LOQ= 1 ng/g.

3.4. Pollen analysis of honey

Microscopic examination of pollen (melissopalynology) showed that all orange honey samples which contained synephrine presented citrus pollen. Usually the amount of pollen in citrus honey samples is low (ESCRICHE et al., 2011; KUS et al., 2015). The sample which was commercialized as orange honey but did not contain detectable levels of synephrine (B), contained predominantly pollen from *Eucalyptos* sp. and did not present citrus pollen. It was considered of poor quality as it also contained intense granulous mass. Citrus pollen was detected in one of the wildflowers samples (S12) which contained 236.5 ng/g of synephrine. All of the orange honey were not pure orange honeys, as other types of pollens were identified in considerable amount

(Figure 4b). Rodríguez et al. (2010) collected, directly from apicultural holdings, 13 samples of citrus honey and found a range of between six and sixteen different pollen types per sample. The picture presented in Figure 4 shows a citrus pollen grain in one of the orange honey samples (a) and other pollen grains in one of the orange honey samples commercialized in Brazil (b).

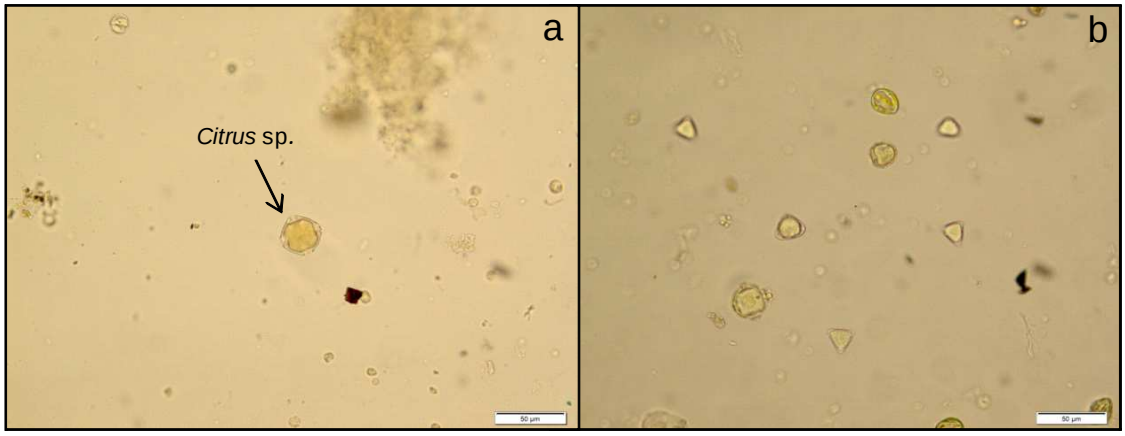

Figure 4. Citrus pollen grain in (a) orange honey and (b) pollen grains in an orange honey sample from the consumer market (b). Bars: 50 µm.

4. Conclusion

A method was optimized for the determination of synephrine in honey. The method involves extraction with 5% trichloroacetic acid, clean up with 30 mg of florisil, analysis in LC-MS/MS and presented recoveries were the acceptable range (74.3-90.7%). The method validated and proved to be suitable for the detection and quantitation of synephrine in honey samples. It presented limits of detection and quantification of 0.66 ng/g and 1 ng/g, respectively, coefficient of variation of repeatability (CV_r) ranged from 1.8% to 4.5% and the CV of reproducibility (CV_R) varied from 3.4% to 7.4%. Synephrine was detected in orange honey at levels varying from 79.2 to 432.2 ng/g; and also in wildflowers honey (9.4 to 236.5 ng/g) with some contribution of citrus flowering. These results were complemented by pollen analysis (melissopalynology), which is traditional approach to recognize the botanical origin of honey. Synephrine was observed to be present in flowers of different citrus species but not in other honey flowers, confirming that it is mainly present in citrus. Based on the results, synephrine would be a reliable authentication index for orange honey and also an indicator of presence of citrus species in honey samples.

CONCLUSÕES INTEGRADAS

A partir desses estudos foi possível concluir que o mel é um alimento que pode ser utilizado como ferramenta de auxílio na determinação da contaminação ambiental por pesticidas. Além disso, a determinação de resíduos de pesticidas em mel é importante no sentido de assegurar que a população não esteja exposta a níveis de contaminações acima dos limites aceitáveis. Dessa forma, o levantamento realizado dos estudos disponíveis na literatura, com o desenvolvimento de métodos analíticos cromatográficos para a determinação de pesticidas em mel, permitiu identificar as limitações existentes, especialmente em relação ao número de compostos analisados e técnicas analíticas empregadas.

QuEChERS e técnicas miniaturizadas, tais como a extração líquido-líquido dispersiva (DLLME), microextração por sorbente empacotado (MEPS) e microextração em fase sólida (SPME) têm sido amplamente utilizadas na etapa de preparo de amostras para determinação de pesticidas em mel, associadas à cromatografia líquida de ultra eficiência acoplada a espectrometria de massas (UHPLC-MS/MS).

Foi desenvolvido um método para determinação multirresíduo de 116 pesticidas em mel utilizando QuEChERS e UHPLC-MS/MS. Foram obtidas recuperações entre 81,6 e 108,9 %, coeficiente de variação menor ou igual a 20% e incerteza expandida de até 48.5%. O limite de detecção (LOD) foi de 0,005 mg/kg e os limites de quantificação (LOQ) foram 0,01 e 0,025 mg/kg. Todos os parâmetros estiveram de acordo com as recomendações da Comunidade Européia para resíduos de pesticidas em alimentos (SANCO n^{o} 12571/2013).

Em geral, as amostras de mel analisadas apresentaram qualidade adequada quanto à presença de pesticidas. Das 100 amostras de mel provenientes de diferentes regiões do Brasil, apenas 1 amostra, esta comercializada no estado de Minas Gerais, apresentou 0,029 mg/kg de triclorfon.

Foi desenvolvido um método por LC-MS/MS para analise de sinefrina, amina bioativa tipicamente encontrada em espécies de citros, como biomarcador de autenticidade de mel de citros. O método consistiu de extração com ácido tricloroacético 5%, clean com Florisil e analise em LC-MS/MS. O método apresentou limites de detecção e quantificação de 0,66 e 1 ng/g, respectivamente, e valores de recuperação entre 74,3 e 90,7%, demonstrando ser adequado ao proposito. Sinefrina foi detectada nas amostras de mel com alguma contribuição de florada de citros e, portanto essa amina pode ser utilizada como um indicativo da presença de espécies de citros em amostras de méis.

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ANEXO A

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Pesticides in honey: A review on chromatographic analytical methods

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ABSTRACT

Honey is a product of high consumption due to its nutritional and antimicrobial properties. However, residues of pesticides, used in plagues' treatment in the hive or in crop fields in the neighborhoods, can compromise its quality. Therefore, determination of these contaminants in honey is essential, since the use of pesticides has increased significantly in recent decades because of the growing demand for food production. Furthermore, pesticides in honey can be an indicator of environmental contamination. As the concentration of these compounds in honey is usually at trace levels and several pesticides can be found simultaneously, the use of highly sensitive and selective techniques is required. In this context, miniaturized sample preparation approaches and liquid or gas chromatography coupled to mass spectrometry became the most important analytical techniques. In this review we present and discuss recent studies dealing with pesticide determination in honey, focusing on sample preparation and separation/detection methods as well as application of the developed methods worldwide. Furthermore, trends and future perspectives are presented.

Contents

Abbreviations: AED, Atomic electron detector; APCI, Atmospheric pressure chemical ionization; ASE, Accelerated solvent extraction; CME-UABE, Coacervative microextraction ultrasound-assisted back-extraction; DD, Double derivatization; DLLME, Dispersive liquid liquid microextraction; dSPE, Dispersive solid phase extraction; ECD, Electron capture detector; ESI, Electrospray ionization; ET, Elevated temperature; Fl, Spectrofluorimetric detector; FPD, Flame photometric detector; GC, Gas chromatography; HPLC, High performance liquid chromatography; HRMS, High resolution mass spectrometry; HS-SPME, Headspace solid phase microextraction; IL, Ionic liquid; IT, Ion trap; IT/MS, Ion trap mass spectrometry; LC/DD/Fl, Liquid chromatography double derivatization coupled with spectrofluorimetric detector; LLE, Liquid liquid extraction; LOD, Limit of detection; LOQ, Limit of quantification; LTP, Low temperature purification; MEPS, Microextraction by packed sorbent; MRL, Maximum residue level; MS, Mass spectrometry; MS/MS, Tandem mass spectrometry; MSPE, Magnetic solid phase extraction; Nd, Not detected; nf, Not found; NPD, nitrogen phosphorus detector; PSA, Primary secondary amine; QuEChERS, Quick, easy, cheap, effective, ruged and safe; SBSE, Stir bar sorptive extraction; SDME, Single drop microextraction; SLE, Solid supported liquid liquid extraction; SPE, Solid phase extraction; SPME, Solid phase microextraction; ToF, time of flight; UA, Ultrasound assisted; UHPLC, Ultra high performance liquid chromatography; UV, Ultraviolet

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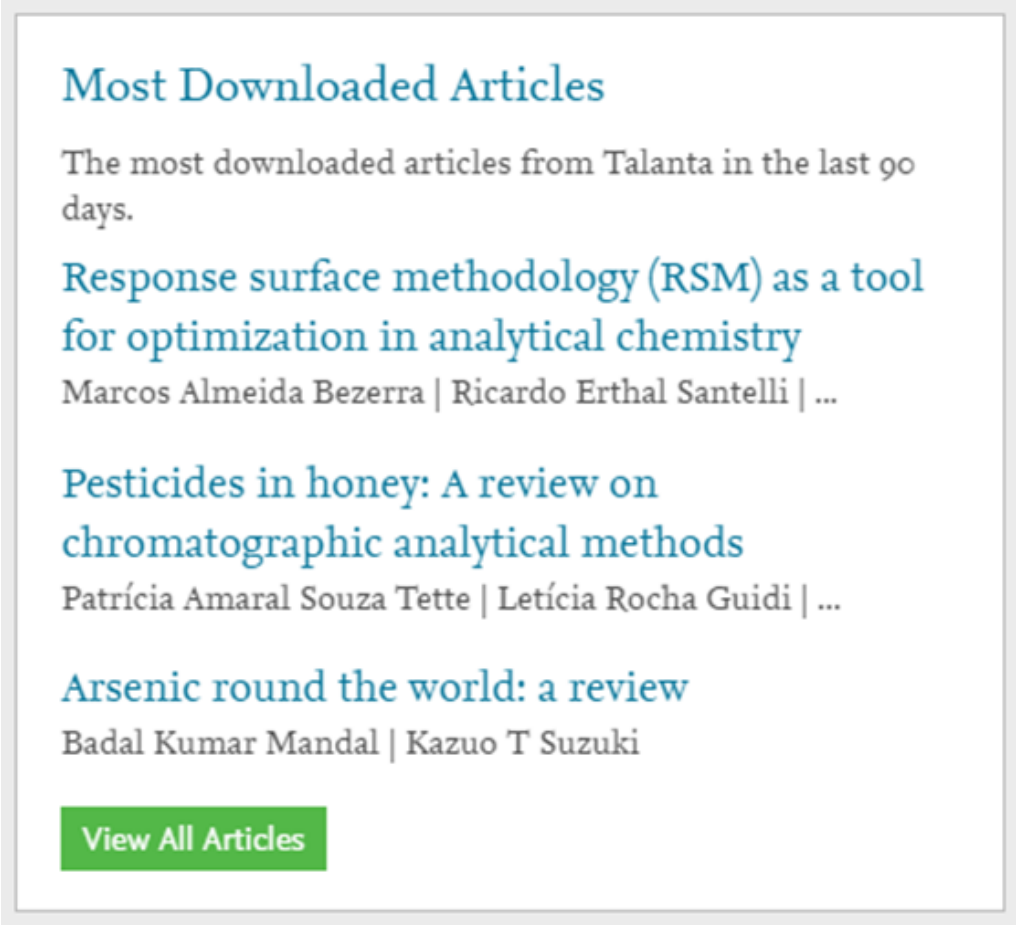
ANEXO B



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ANEXO C

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Analytical Methods

Multiclass method for pesticides quantification in honey by means of modified QuEChERS and UHPLC–MS/MS

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ABSTRACT

Bee products can be produced in an environment contaminated by pesticides that can be transported by honey bees to the hive and incorporated into the honey. Therefore, rapid and modern methods to determine pesticide residues in honey samples are essential to guarantee consumers' health. In this study, a simple multiresidue method for the quantification of 116 pesticides in honey is proposed. It involves the use of a modified QuEChERS procedure followed by UHPLC–MS/MS analysis. The method was validated according to the European Union SANCO/12571/2013 guidelines. Acceptable values were obtained for the following parameters: linearity, limit of detection (0.005 mg/kg) and limit of quantification (0.010 and 0.025 mg/kg), trueness (for the four tested levels the recovery assays values were between 70 and 120%), intermediate precision (RSD < 20.0%) and measurement uncertainty tests (<50.0%). The validated method was applied for determination of 100 honey samples from five states of Brazil.

1. Introduction

Honey is one of the most used products of the hive, both naturally and in several industrialized forms (Komatsu, Marchini, & Moreti, 2002). Known since ancient times, honey has always attracted the attention of man, especially because of its sweet taste (Bera & Almeida-Muradian, 2007; Rossi, Martinelli, Lacerda, Camargo, & Victória, 1999). Furthermore, several hive products have been appreciated due to their antimicrobial and antiseptic properties. However, in recent years, the pesticide monitoring in honey has become a public health issue in view of the growth of the levels of these chemicals in bee products (Li et al., 2013; Rial-Otero, Gaspar, Moura, & Capelo, 2007). Therefore, the monitoring of pesticide residues in honey is important to evaluate the potential risk of these products to consumers' health. Also, such monitoring can provide information about the use of pesticides in crop fields around the hives and in their neighborhoods. In this case, honey can be used as a bio-indicator for the evaluation of environmental impact (Rissato, Galhiane, Knoll, Andrade, & Almeida, 2006).

In this context, analytical methods for the determination of pesticides in honey must be available for routine analysis. The determination of pesticide residues in foods requires a prior step of sample preparation due to the low concentrations of the analytes in the sample, the distinct chemical properties of the analytes and the complexity of the matrices (Prestes, Friggi, Adaime, & Zanella, 2009). Although most of these procedures are carried out by conventional techniques, such methods are generally not applicable to all food matrices, do not produce clean extracts and generate low recovery. These disadvantages have led to the development of new approaches with an emphasis on the practicality of implementation, the use of significantly lower amounts of organic solvents, and the ability to detect analytes in very low concentrations. In recent years, efforts in the field of analytical chemistry focused on the miniaturization of sample preparation associated with improvement in selectivity and sensitivity (Melwanki & Fuh, 2008). However, these efforts are far from being considered ideal, due to the limitation of application, quickness, sensitivity and reliability of the results (Martínez-Vidal, Liébanas, Rodríguez, Frenich, & Moreno, 2005). In this context, QuEChERS

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