Food Chemistry 229 (2017) 527–533

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Synephrine – A potential biomarker for orange honey authenticity

Patrícia A.S. Tette [a], Letícia R. Guidi [a], Esther M.A.F. Bastos [b], Christian Fernandes [a,c], Maria Beatriz A. Gloria [a,*]

[a] LBqA – Laboratório de Bioquímica de Alimentos, Faculdade de Farmácia, Universidade Federal de Minas Gerais, Av. Antonio Carlos 6627, bloco 3, sala 2091, Pampulha, CEP 31210-901 Belo Horizonte, MG, Brazil
[b] Serviço de Recursos Vegetais e Opoterápicos, Fundação Ezequiel Dias, Rua Conde Pereira Carneiro, 80, Gameleira, CEP 30510-010 Belo Horizonte, MG, Brazil
[c] Laboratório de Controle de Qualidade de Medicamentos e Cosméticos, Faculdade de Farmácia, Universidade Federal de Minas Gerais, Av. Antonio Carlos 6627, bloco 2, sala 4029, Pampulha, CEP 31210-901 Belo Horizonte, MG, Brazil

ARTICLE INFO

Article history:
Received 2 November 2016
Received in revised form 2 February 2017
Accepted 21 February 2017
Available online 23 February 2017

Chemical compound studied in this manuscript:
Synephrine (PubChem CID: 7172)

Keywords:
Synephrine
Orange honey
Citrus honey
Honey
Amines
Botanical origin
Biomarker

ABSTRACT

A LC-MS/MS method for synephrine as a biomarker for orange honey authenticity was developed and validated. The sample was extracted with 5% TCA and cleaned up with Florisil providing 83.7% recoveries. Ions transitions for quantification and identification were 168 → 135.0 and 168 → 107.0, respectively. The limits of detection and quantification were 0.66 and 1.0 ng/g, respectively. Synephrine was detected in orange honey at levels from 79.2 to 432.2 ng/g, but not in other monofloral honeys. It was also present in some wildflower honeys (9.4–236.5 ng/g), showing contribution of citrus to this polyfloral honey. Results were confirmed by qualitative pollen analysis. No citrus pollen was detected in honey containing synephrine levels ≤43.8 ng/g, suggesting that synephrine in honey is more sensitive compared to pollen analysis. Synephrine was found in citrus but not in other apiculture flowers. Therefore, synephrine is a botanical marker to differentiate and attest authenticity of orange honey.

1. Introduction

Honey is a natural food known for its nutritional and medicinal value. It is often used as a sugar substitute due to its sweetness, desirable color and flavor characteristics. It is also utilized as ingredient or natural preservative in many foods (Pyrzynska & Biesaga, 2009). Chemically, honey is composed of a mixture of sugars, including monosaccharides (75%), disaccharides (10–15%) and small amounts of other sugars (Silva, Gauche, Gonzaga, Costa, & Fett, 2016). Other components are also present in minor proportions, such as minerals (calcium, copper, iron, magnesium, phosphorus, and potassium), proteins, amino acids, vitamins, flavonoids, pigments, and several organic acids. Among components, several show antioxidant properties including chrysin, pinobanksin, vitamin C, catalase and pinocembrine (Blasco, Vazquez-Roig, Onghena, Masia, & Picó, 2011; Downey, Hussey, Kelly, Walshe, & Martin, 2005; Fallico, Zappala, Arena, & Verzera, 2004; Finola, Lasagno, & Marioli, 2007; Silva et al., 2016).

The quality of honey can be affected by several factors including types of bees, presence of sucking insects, botanical origin, geographical location, climatic conditions, ripening stage, as well as processing and storage conditions. For this reason, honey may show different consistency, color, flavor and aroma (Downey et al., 2005; Komatsu, Marchini, & Moreti, 2002; Silva et al., 2016).

The most common types of plants used for honey production are eucalyptus, citrus and wildflowers (Komatsu et al., 2002). Honey can be produced from the nectar of a single botanical species - monofloral - or more than one species - polyfloral (Bastos, Franco, Silva, Janzantti, & Marques, 2002). Generally, a monofloral honey has defined aroma and taste which makes it especially appreciated by consumers (Fallico et al., 2004).

Citrus honey is considered one of the best monofloral honeys. In addition to the appreciated flavor, the floral fragrance is exclusive of this type of honey. It is also quite popular. It is characterized by a light color, intense odor, mild flavor and fine crystallization

* Corresponding author.
E-mail address: mbeatriz@ufmg.br (M.B.A. Gloria).

http://dx.doi.org/10.1016/j.foodchem.2017.02.108
0308-8146/

(Komatsu et al., 2002; Sesta, Piana, Oddo, Lusco, & Belligoli, 2008; Terrab, Díez, & Heredia, 2003).

Evaluation of honey authenticity is important in the context of consumer protection, quality control and trade purposes (Verzera, Tripodi, Condurso, Dima, & Marra, 2014). The most commonly used approach is melissopalynological analysis, which consists in the botanical classification of honey by identification and quantification of the percentage of pollen under microscopic examination. Although this method is widely used, it has shown limitations for citrus honey, because its pollen is considered 'under represented' (Escriche, Kadar, Juan-Borrás, & Domenech, 2011; Kus & Ruth, 2015; Rodriguez, Salud, Hortensia, Luis, & Jodral, 2010). When compared to other honeys, the amount of pollen present in citrus honey is lower, considering the strongly present characteristics of flavor and taste (Escriche et al., 2011; Rodriguez et al., 2010). Chemical components can also be used to discriminate honeys from different botanical origins. Several classes of compounds can be botanical markers, among them, phenolics, flavonoids, organic acids, terpenes, sugars, amino acids, among others (Boffo, Tavares, Tobias, Ferreira, & Ferreira, 2012; Escriche et al., 2011; Ferreres, Viguera, Lorente, & Barberán, 1993; Liang, Cao, Chen, Xiao, & Zheng, 2009; Schievano, Morelato, Facchin, & Mammi, 2013; Serrano, Villarejo, Espejo, & Jodral, 2004; Verzera et al., 2014). Some compounds have been suggested as markers of orange honey authenticity, such as caffeine, (*E*)-2,6-dimethylocta-2,7-diene-1,6-diol (Schievano et al., 2013), hesperetin (Escriche et al., 2011; Ferreres et al., 1993; Liang et al., 2009); naringenin and caffeic acid (Escriche et al., 2011). Although these compounds are important for characterization of citrus honeys, they are complex, may require long analysis time, and are not specific of orange honey, and could be present in other monofloral honeys. Furthermore, they require confirmation by other techniques.

Synephrine is an aromatic amine, characteristic of citrus. It has been successfully used as a biomarker for authenticity of orange juice and orange soft drink (Stewart & Wheaton, 1964; Vieira, Silva, & Gloria, 2010). Synephrine is a sympathomimetic amine. It can cause vasoconstriction, increased blood pressure and relaxation of the bronchial muscle. It is also useful in reducing fat mass in obese humans as it stimulates lipolysis and raises metabolic rate and oxidation of fat through increased thermogenesis (Kusu, Matsumoto, Arai, & Takamura, 1996; Stewart, Newhall, & Edwards, 1964; Tsujita & Takaku, 2007; Vieira, Theodoro, & Gloria, 2007; Vieira et al., 2010). Because of beneficial properties associated with this compound, the identification of synephrine in honey would be a value-added feature. Therefore, the aim of this study was to develop and validate a LC-MS/MS method for the analysis of synephrine in orange honey and to evaluate its use as an authenticity index for this monofloral honey.

2. Experimental

2.1. Honey samples

Monofloral honey samples from *Apis mellifera* bees, including citrus (*Citrus* sp., eight different brands, n = 8), 'assa-peixe' or vernonia (*Vernonia* sp., n = 2), and eucalyptus (*Eucalyptus* sp., n = 3) were purchased from the consumer's market and 'aroeira' honey (*Myracrodruon urundeuva*, n = 5) was provided by Serviço de Recursos Vegetais e Opoterápicos (SRVO, FUNED). Wildflower (polyfloral) honeys (16 different brands) were also purchased from the consumer's market of São Paulo and Minas Gerais, Brazil, eight different brands from each state. Eucalyptus honey was used as blank during method development. The samples were stored at room temperature (20 °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. Liquid 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 × 2.0 mm, 3 μm) 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 °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 at 20 psi, collision gas at 4 psi, nebulizer gas and auxiliary gas at 20 psi and ion source temperature of 500 °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.

2.4. Sample preparation

The study was undertaken at Laboratório de Bioquímica de Alimentos – LBqA, UFMG, which is accredited by INMETRO (National Institute of Metrology, Quality and Technology) according to ISO 17025:2005 for the analysis of histamine in fish. 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 0.1 M HCl for synephrine and L-norvaline (internal standard), respectively. Honey samples (1 g) were weighed in 50 mL tubes and spiked with proper amounts of working standard solution of synephrine and of the internal standard norvaline (10 ng/mL). Afterwards, 9 mL of 5% trichloroacetic acid (TCA) was added and the sample was vortexed (Velp Scientifica, Wizard, Usmate, Italy), sonicated (LS Logen Scientific, Diadema, São Paulo, Brazil) and centrifuged (Jouan MR23I, Saint Herblain, France). The supernatant was filtered through qualitative paper. Filtrates were collected and the volume was brought up to 10 mL in calibrated volumetric flasks. An aliquot (1 mL) was transferred to 2 mL tube containing Florisil and PSA for clean-up, and submitted to vortex and centrifugation (Eppendorf 5424R, Hauppauge, New York, NY, USA). Finally, the extracts were filtered through qualitative filter paper and 0.45 μm 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 synephrine recovery 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 × *g*), relative centrifugal force (X_3 = 1000, 11,000 and 21,000 × *g*), centrifugation

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.

time (X_4 = 2, 6 and 10 min) and sonication time (X_5 = 0, 15 and 30 min).

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 synephrine recoveries. The variables considered were centrifugation time (min), ultrasound time (min) and vortexing time (s). The temperature and relative centrifugal force were set at 4 °C and 11,000 × *g*, respectively, and vortexing speed was set at 300 × *g*. The experimental values and coded levels for the independent variables used in the CCRD are indicated in Table 2. The optimized extraction conditions that provided the best recoveries were confirmed using six replicates.

2.4.2. Optimization of the variables affecting synephrine clean up

Another Plackett-Burman design was used to screen the factors which could affect clean up of synephrine extracts. The design was similar to that used for the extraction procedure and included 12 tests, six repetitions at the central point and five independent variables. The variables were amount of sorbents (X_1 = 20, 50 and 80 mg), type of sorbents (X_2 = Florisil and Florisil with PSA), vortexing time (X_3 = 20, 30 and 40 s), centrifugation speed (X_4 = 1000, 9000 and 17,000 × *g*) and centrifugation time (X_5 = 1, 5 and 9 min). When two sorbents were used, the same amount of each was used.

Then, a Central Composite Rotational Design (CCRD) was performed based on Plackett-Burman results. This experiment was conducted with three replications at the central point to optimize synephrine recoveries from honey. The variables were amount of sorbent (mg), centrifugation speed (*g*) and centrifugation time (min). Centrifugation temperature and vortexing time were set at 22 °C and 10 s, respectively. Experimental values and coded levels for the independent variables used in the CCRD are also presented in Table 2. The optimized extraction conditions that provided the best recoveries were confirmed using six replicates.

2.5. Method validation

Validation was performed following the European Commission guidelines (EC European Commission, 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 correlation coefficients 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. The method was also applied in the analysis of four apiculture flowers (*Vernonia polyanthes*, *Montanoa pyramidata*, *Tithonia diversifolia* and *Clerodendron speciosum*), which are widely available in Brazil. The analyses were performed in triplicate.

2.7. Pollen analysis

Qualitative melissopalynology was performed according to Belay, Solomon, Bultossa, Adgaba, and Melaku (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

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 and clean up of synephrine from honey.

Independent variables	Code units	Coded variable levels				
		−1.68	−1	0[a]	+1	+1.68
Extraction						
Centrifugation time (min)	X_1	1	4	8	12	15
Ultrasonication time (min)	X_2	1	7	15.5	24	30
Vortexing time (s)	X_3	40	60	90	120	140
Clean up						
Amount of sorbent (mg)	X_1	13	30	55	80	97
Centrifugation speed (g)	X_2	1000	5000	11,000	17,000	21,000
Centrifugation time (min)	X_3	1	3	5.5	8	10

[a] Center point.

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 a microscope (Olympus BX50, Hamburg, Germany). The samples were identified using reference slides. The analysis was undertaken at Serviço de Recursos Vegetais e Opoterápicos at 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 procedure

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, 2015). The concentration of 5% TCA was used in several studies to extract amines from food (Bandeira, Evangelista, & Gloria, 2012; Evangelista et al., 2016; Guidi & Gloria, 2012; Paiva, Evangelista, Queiroz, & Gloria, 2015).

The recoveries from the Plackett-Burman design ranged from 72.8 to 83.3%. Only centrifugation time affected the results ($p = 0.012$) (Fig. 1a). The significance level of 0.1 was used to avoid exclusion of important variables in the subsequent design (Rigueira, Rodrigues, & Gloria, 2011). Since centrifugation time affected significantly synephrine recovery, it was further investigated in the CCRD along with ultrasonication and vortexing times. The centrifugation and vortexing speeds were set at 11,000 × *g* and 300 × *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% TCA, 4 min centrifugation time, 11,000 × *g* centrifugation speed, centrifugation temperature of 4 °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 4.6% coefficient of variation. The recoveries obtained are acceptable according to the European Union guidelines (EC, 2002).

A second Plackett-Burman design was used to optimize sample clean up by means of dispersive solid phase extraction employing PSA and Florisil. 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., 2016). 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 ($p = 0.005$) affected recovery (Fig. 1b). Pareto chart showed that the amount of sorbent had a more important effect compared to the type of sorbent.

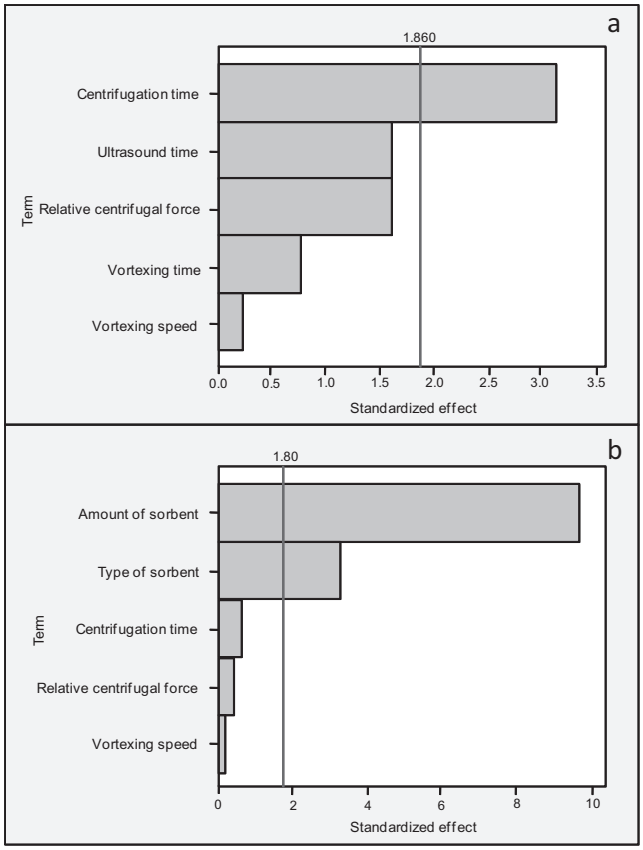

Fig. 1. Pareto charts obtained in the optimization of (a) the extraction procedure and (b) clean-up by means of Plackett-Burman design for LC-MS/MS analysis of synephrine in honey.

Based on these results, the subsequent design was performed only with Florisil and speed and time of centrifugation were set at 17,000 × *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 × *g* centrifugation speed, 5 min centrifugation time and 22 °C centrifugation temperature. These conditions provided average recovery from 83.7 ± 6.6% and 7.9% coefficient of variation.

3.2. Method validation

The analytical matrix-matched calibration curves for synephrine were linear within the range of 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., 2016).

According to Table 3, the average accuracy (n = 18) determined at three different concentrations was 79.7%. The coefficient of vari-

Table 3
Precision and accuracy of optimized method for the analysis of synephrine in honey by LC-MS/MS.

Concentration (ng/mL)		Precision (%)		Accuracy
Nominal	Experimental (mean ± sd)	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.

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

The specificity of the method was verified by analyzing the chromatograms (Fig. 2). Two ions transitions for quantification and identification (168 → 135.0 and 168 → 107.0, respectively) 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). The LOD and LOQ were 0.66 ng/g and 1.0 ng/g, respectively.

3.3. Application of the method to honey and flowers

3.3.1. Synephrine in monofloral honeys

The optimized and validated method was used in the analysis of the most relevant monofloral honey available in the consumer's market. Synephrine was found in seven orange honeys (n = 8), but not in other types of monofloral honeys analyzed – eucalyptus, vernonia and myracrodruon (Table 4).

The concentration of synephrine in the different brands of orange honey ranged widely, from 79.2 to 432.2 ng/g. Synephrine is one among other bioactive amines found in orange juice; however, it is characteristic of citrus species and it is not commonly detected in other food products (Gloria, 2005). In fact, Vieira et al. (2010) 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.

Besides its relevance as an authenticity biomarker, the presence of synephrine in orange honey is also interesting from a human health stand point. Therefore, the presence of synephrine in orange honey can add value to the product.

3.3.2. Synephrine in wildflower honeys

Wildflower honey from two states which are relevant orange producers in Brazil were also analyzed for synephrine (Table 4). Among samples from the state of Minas Gerais (n = 8), synephrine was detected in one brand, at low concentration (mean level of 22.0 ng/g). However, six out of eight samples from the state of São Paulo, contained synephrine at mean concentrations ranging from 9.4 to 236.5 ng/g. This result suggests the contribution of orange to wildflower honey from São Paulo. In fact, São Paulo is the main producer of orange in Brazil, concentrating the highest numbers of orange trees (Brasil. Instituto Brasileiro de Geografia e Estatística., 2016a). Many plant species can contribute with the nectar of polyfloral honeys; however, geographical location of beehive plays a major role. Every day during pollination of agricultural crops, 10,000–25,000 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, Galhiane, Almeida, Gerenutti, & Apon, 2007).

3.3.3. Synephrine in citrus and other apiculture flowers

To make sure that synephrine would be a reliable index of authenticity of citrus honey, it is important to ascertain that it is present in citrus flowers but absent in others common bee flowers. Although the method has been developed for honey, it was also used in the analysis of flowers from different citrus species and also in other four common apiculture flowers. According to Table 4, all citrus flowers contained synephrine, at mean concentrations ranging from 0.055 to 1932.6 ng/g. The largest concentrations were found in *Citrus reticulata* (Blanco) followed by *Citrus sinensis* (L. Osbeck). This last one is the main citrus species produced in Brazil (Brasil. Ministério da Agricultura Pecuária e Abastecimento., 2016b; Vieira et al., 2007). Synephrine was not detected in other relevant apiculture flowers (*Vernonia polyanthes*, *Montanoa pyramidata*, *Tithonia diversifolia* and *Clerodendron speciosum*) and this reinforces its applicability as an authenticity biomarker.

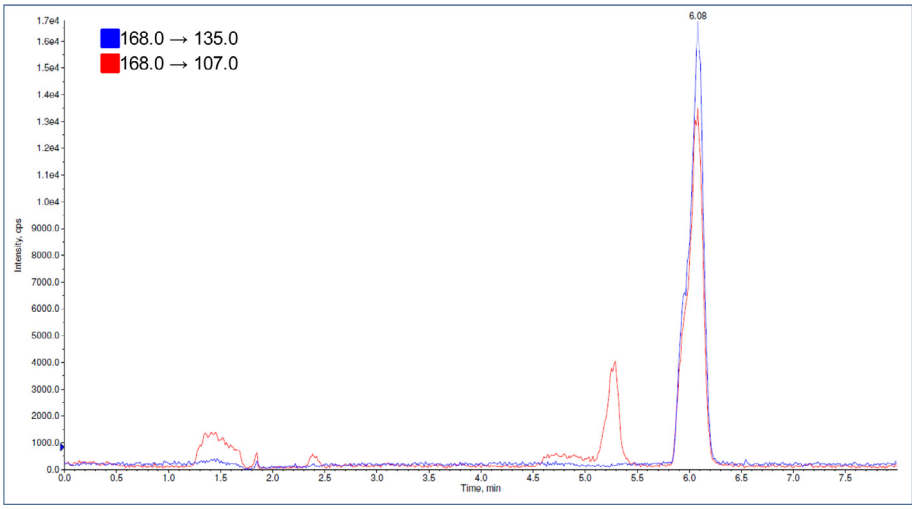

Fig. 2. Total ion chromatograms (TIC) obtained by HPLC–MS/MS (ESI positive mode) for blank honey extracts spiked with synephrine at 9 ng/mL.

Table 4
Synephrine levels and presence of orange pollen in monofloral honeys [orange (*Citrus* sp.), *Eucalyptus* sp., *Vernonia* sp. and *Myracrodruon urundeuva*], and polyfloral – wildflower honeys from Minas Gerais and São Paulo, Brazil and synephrine levels in citrus and other apiculture flowers.

Samples Honeys	Synephrine[a] (ng/g)	Orange pollen[‡] (presence)
Monofloral honey		
Orange (*Citrus* sp.)		
A	166.3 ± 2.3	+
B	nd[†]	–
C	79.2 ± 3.0	+
D	227.3 ± 2.9	+
E	218.6 ± 3.2	+
F	177.6 ± 2.2	+
G	304.6 ± 2.9	+
H	432.2 ± 3.6	+
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	–
Polyfloral honey		
Wildflower honey (Minas Gerais)		
S1 and S8	<LOQ	–
S2, S3, S4, S5 and S7	nd	–
S6	22.0 ± 0.7	–
Wildflower honey (São Paulo)		
S9 and S15	nd	–
S10	43.8 ± 0.7	–
S11	9.5 ± 0.1	–
S12	236.5 ± 2.6	+
S13	29.0 ± 1.7	–
S14	9.4 ± 0.1	–
S16	31.3 ± 1.9	–
Citrus flowers	(μg/g)	- -[‡]
Citrus sinensis L. Osbeck – 'Bahia'		
A	813.0 ± 8.0	- -
B	523.5 ± 78.7	- -
Citrus sinensis L. Osbeck – 'Serra d'água'		
A	989.5 ± 51.0	- -
B	1090.1 ± 111.6	- -
Citrus limetta		
A	585.5 ± 17.6	- -
B	684.1 ± 47.3	- -
Citrus latifolia (Tanaka)		
A	0.057 ± 0.002	- -
B	0.055 ± 0.002	- -
Citrus reticulata (Blanco)		
A	1932.6 ± 102.2	- -
Other apiculture flowers	(μg/g)	- -
Vernonia polyanthes	nd	- -
Montanoa pyramidata	nd	- -
Tithonia diversifolia	nd	- -
Clerodendron speciosum	nd	- -

‡ – = not present; + = present; - - = not applicable.
[a] Mean value ± standard deviation.
[†] nd = not detected (LOQ = 1 ng/g).

According to Schievano et al. (2013), the identification of markers of botanical origin of honey is important and can lead to the formation of a database, which can be used to classify honeys of different sources. Such markers can also provide an alternative method to melissopalynological analysis.

3.4. Pollen analysis of honey

3.4.1. Pollen analysis of orange honey

Microscopic examination of pollen (melissopalynology) showed that every orange honey sample which contained synephrine also contained citrus pollen (Table 4). Citrus pollens were present even though, according to the literature, citrus pollen is usually underrepresented in citrus honey (Escriche et al., 2011; Kus & Ruth, 2015).

No citrus pollen was identified in one brand of orange honey (brand B), which did not contain detectable levels of synephrine. In this sample, pollen from *Eucalyptus* sp. was identified. Microscopic analysis also showed intense granulous substances suggesting poor quality honey. Based on this result, this sample was erroneously identified as orange honey.

All of the orange honeys were not pure, as other types of pollen were also identified in the samples. In a similar way, Rodriguez et al. (2010) found 6 to 16 different types of pollen in citrus honey (n = 13) collected directly from apicultural holdings. Therefore, it is likely that citrus honey will have predominantly, but not exclusively, citrus pollen.

3.4.2. Pollen analysis of wildflower honey

Citrus pollen was also identified in one of the wildflower samples (S12) which contained 236.5 ng/g synephrine. In the other samples, which had synephrine at levels ≤43.8 ng/g, no citrus pollen was detected. This suggests that at citrus contributions to honey leading to synephrine levels ≤43.8 ng/g, no citrus pollen can be identified. And thus, it implies that the determination of synephrine in honey is more sensitive and reliable compared to pollen analysis in the determination of the presence of citrus in honey.

Based on these results, authentic orange honey contained synephrine at levels ranging from 79.2 to 432.2 ng/g and citrus pollen was present. Wildflower honey, especially from São Paulo, also contained synephrine, showing the contribution of citrus to wildflower honey in this state. Indeed São Paulo is the largest citrus producer in Brazil.

These results confirm the relevance of synephrine as a biomarker for orange honey. Further studies are needed to ascertain if synephrine concentration could be associated with degree of purity of honey.

4. Conclusion

A method was optimized for the determination of synephrine in honey. It involves extraction with 5% trichloroacetic acid, clean up with 30 mg of Florisil and analysis by LC-MS/MS. The method was validated and proved to be suitable for the detection and quantitation of synephrine in honey samples. Recoveries were within the acceptable range (74.3–90.7%). The limits of detection and quantification were 0.66 and 1.0 ng/g, respectively; the coefficient of variation of repeatability (CV_r) ranged from 1.8 to 4.5% and that 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 but not in other monofloral honeys (eucalyptus, vernonia and myracrodruon). It was also present in wildflowers honey (9.4–236.5 ng/g) showing contribution of citrus to this type of honey. These results were confirmed by qualitative pollen analysis (melissopalynology), which is the traditional approach to recognize the botanical origin of honey. Synephrine was present in flowers of different citrus species but not in other honey flowers, confirming that it is mainly present in citrus. Based on these results, synephrine would be a reliable authentication index for orange honey, indicating the presence of citrus species in honey samples. The method is fast, efficient and can be used as an additional approach for the identification of the botanical origin of orange honey.

Acknowledgements

The authors acknowledge Coordenação de Pessoal de Nível Superior – CAPES, Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq (Brasília, DF, Brazil) and Fundação de Amparo a Pesquisa do Estado de Minas Gerais – FAPEMIG (Belo

Horizonte, MG, Brazil) for the financial support. The authors also thank Edineia Souza Xavier and Rânia Mara Santana for technical support.

References

Bandeira, C. M., Evangelista, W. P., & Gloria, M. B. A. (2012). Bioactive amines in fresh, canned and dried sweet corn, embryo and endosperm and germinated corn. *Food Chemistry, 131*, 1355–1359.

Bastos, D. H. M., Franco, M. R. B., Silva, M. A. A. P., Janzantti, N. S., & Marques, M. O. M. (2002). Composição de voláteis e perfil de aroma e sabor de méis de eucalipto e laranja. *Ciência e Tecnologia de Alimentos, 22*, 122–129.

Belay, A., Solomon, W. K., Bultossa, G., Adgaba, N., & Melaku, S. (2015). Botanical origin, colour, granulation, and sensory properties of the Harenna forest honey, Bale, Ethiopia. *Food Chemistry, 167*, 213–219.

Blasco, C., Vazquez-Roig, P., Onghena, M., Masia, A., & Picó, Y. (2011). Analysis of insecticides in honey by liquid chromatography-ion trap-mass spectrometry: Comparison of different extraction procedures. *Journal of Chromatography A, 1218*, 4892–4901.

Boffo, E. F., Tavares, L. A., Tobias, A. C. T., Ferreira, M. M. C., & Ferreira, A. G. (2012). Identification of components of Brazilian honey by ^{1}H NMR and classification of its botanical origin by chemometric methods. *LWT – Food Science and Technology, 49*, 55–63.

Brasil. Instituto Brasileiro de Geografia e Estatística. (2016a). Levantamento Sistemático da Produção Agrícola. <http://www.ibge.gov.br/home/estatistica/indicadores/Agrope-cuaria/lspa/defaulttab.shtm> Accessed 05.08.16.

Brasil. Ministério da Agricultura Pecuária e Abastecimento. (2016b). Citrus. <http://www.agricultura.gov.br/vegetal/culturas/citrus>. Accessed 21.09.16.

Downey, G., Hussey, K., Kelly, J. D., Walshe, T. F., & Martin, P. G. (2005). Preliminary contribution to the characterisation of artisanal honey produced on the island of Ireland by palynological and physicochemical data. *Food Chemistry, 91*, 347–354.

EC European Commission (2002). Commission Decision 2002/657/EC of 12 August: Implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Official Journal of European Communities, 221*, 8–36.

Escriche, I., Kadar, M., Juan-Borrás, M., & Domenech, E. (2011). Using flavonoids, phenolic compounds and headspace volatile profile for botanical authentication of lemon and orange honeys. *Food Research International, 44*, 1504–1513.

Evangelista, W. P., Silva, T. M., Guidi, L. R., Tette, P. A. S., Byrro, R. M. D., Santiago-Silva, P., ... Gloria, M. B. A. (2016). Quality assurance of histamine analysis in fresh and canned fish. *Food Chemistry, 211*, 100–106.

Fallico, B., Zappala, M., Arena, E., & Verzera, A. (2004). Effects of conditioning on HMF content in unifloral honeys. *Food Chemistry, 85*, 305–313.

Fernandes, C., & Gloria, M. B. A. (2015). Bioactive amines. In L. L. Nollet & F. Toldra (Eds.) *Handbook of food analysis* (3rd ed.) (Vol. 2, pp. 301–328).

Ferreres, F., Viguera, C. G., Lorente, F. T., & Barberán, F. A. T. (1993). Hesperetin: A marker of the floral origin of citrus honey. *Journal of the Science of Food and Agriculture, 61*, 121–123.

Finola, M. S., Lasagno, M. C., & Marioli, J. M. (2007). Microbiological and chemical characterization of honeys from central Argentina. *Food Chemistry, 100*, 1649–1653.

Gloria, M. B. A. (2005). Bioactive amines. In H. Y. Hui & F. Sherkat (Eds.), *Handbook of food science, technology and engineering* (pp. 3632). London: CRC Press.

Guidi, L. R., & Gloria, M. B. A. (2012). Bioactive amines in soy sauce: Validation of method, occurrence and potential health effects. *Food Chemistry, 133*, 323–328.

Komatsu, S. S., Marchini, L. C., & Moreti, A. C. C. C. (2002). Análises físico-químicas de amostras de méis de flores silvestres, de eucalipto e de laranjeira, produzidos por *Apis mellifera* L., 1758 (*Hymenoptera, Apidae*) no estado de São Paulo. 2. Conteúdo de açúcares e de proteína. *Ciência e Tecnologia de alimentos, 22*, 143–146.

Kujawski, M. W., Barganska, Z., Marciniak, K., Miedzianowska, E., Kujawski, J. K., Slebioda, M., & Namiesnik, J. (2014). Determining pesticide contamination in honey by LC-ESI-MS/MS e comparison of pesticide recoveries of two liquid-liquid extraction based approaches. *LWT – Food Science and Technology, 56*, 517–523.

Kus, P. M., & Ruth, S. V. (2015). Discrimination of Polish unifloral honeys using overall PTR-MS and HPLC fingerprints combined with chemometrics. *LWT – Food Science and Technology, 62*, 69–75.

Kusu, F., Matsumoto, M., Arai, K., & Takamura, K. (1996). Determination of synefrine enantiomers in food and conjugated synefrine in urine by high-performance liquid chromatography with electrochemical detection. *Analytical Biochemistry, 235*, 191–194.

Liang, Y., Cao, W., Chen, W., Xiao, X., & Zheng, J. (2009). Simultaneous determination of four phenolic components in citrus honey by high performance liquid chromatography using electrochemical detection. *Food Chemistry, 114*, 1537–1541.

Paiva, C. L., Evangelista, W. P., Queiroz, V. A. V., & Gloria, M. B. A. (2015). Bioactive amines in sorghum: Method optimisation and influence of line, tannin and hydric stress. *Food Chemistry, 173*, 224–230.

Pyrzynska, K., & Biesaga, M. (2009). Analysis of phenolic acids and flavonoids in honey. *Trends in Analytical Chemistry, 28*, 892–902.

Rigueira, J. C. S., Rodrigues, M. I., & Gloria, M. B. A. (2011). Optimization of the analytical extraction of polyamines from milk. *Talanta, 86*, 195–199.

Rissato, S. R., Galhiane, M. S., Almeida, M. V., Gerenutti, M., & Apon, B. M. (2007). Multiresidue determination of pesticides in honey samples by gas chromatography–mass spectrometry and application in environmental contamination. *Food Chemistry, 101*(1719–1726), 2007.

Rodriguez, I., Salud, S., Hortensia, G., Luis, U. J., & Jodral, M. (2010). Characterisation of Sierra Morena citrus blossom honey (*Citrus* sp.). *International Journal of Food Science and Technology, 45*, 2008–2015.

Schievano, E., Morelato, E., Facchin, C., & Mammi, S. (2013). Characterization of markers of botanical origin and other compounds extracted from unifloral honeys. *Journal of Agricultural and Food Chemistry, 61*, 1747–1755.

Serrano, S., Villarejo, M., Espejo, R., & Jodral, M. (2004). Chemical and physical parameters of Andalusian honey: Classification of Citrus and Eucalyptus honeys by discriminant analysis. *Food Chemistry, 87*, 619–625.

Sesta, G., Piana, M. L., Oddo, L. P., Lusco, L., & Belligoli, P. (2008). Methyl anthranilate in Citrus honey. Analytical method and suitability as a chemical marker. *Apidologie, 39*, 334–342.

Silva, P. M., Gauche, C., Gonzaga, L. V., Costa, A. C. O., & Fett, R. (2016). Honey: Chemical composition, stability and authenticity. *Food Chemistry, 196*, 309–323.

Stewart, I., Newhall, W. F., & Edwards, G. J. (1964). The isolation and identification of L-synephrine in the leaves and fruit of citrus. *Journal of Biological Chemistry, 239*, 930–932.

Stewart, I., & Wheaton, T. A. (1964). Phenolic amines in citrus juice. *Proceedings of Florida State Horticultural Society, 77*, 318–320.

Terrab, A., Díez, M. J., & Heredia, F. J. (2003). Palynological, physico-chemical and colour characterization of Moroccan honeys. II. Orange (*Citrus* sp.) honey. *International Journal of Food Science and Technology, 38*, 387–394.

Tette, P. A. S., Oliveira, F. A. S., Pereira, E. N. C., Silva, G., Gloria, M. B. A., & Fernandes, C. (2016). Multiclass method for pesticides quantification in honey by means of modified QuEChERS and UHPLC–MS/MS. *Food Chemistry, 211*, 130–139.

Tsujita, T., & Takaku, T. (2007). Lipolysis induced by segment wall extract from satsuma mandarin orange (*Citrus unshu* Mark). *Journal of Nutrition Science and Vitaminology, 53*, 547–551.

Verzera, A., Tripodi, G., Condurso, C., Dima, G., & Marra, A. (2014). Chiral volatile compounds for the determination of orange honey authenticity. *Food Control, 39*, 237–243.

Vieira, S. M., Silva, T. M., & Gloria, M. B. A. (2010). Influence of processing on the levels of amines and proline and on the physico-chemical characteristics of concentrated orange juice. *Food Chemistry, 119*, 7–11.

Vieira, S. M., Theodoro, K. H., & Gloria, M. B. A. (2007). Profile and levels of bioactive amines in orange juice and orange soft drink. *Food Chemistry, 100*, 895–903.