Journal of Chromatography A, 1426 (2015) 161–173

Contents lists available at ScienceDirect

Journal of Chromatography A

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

A sensitive and efficient method for routine pesticide multiresidue analysis in bee pollen samples using gas and liquid chromatography coupled to tandem mass spectrometry

P. Parrilla Vázquez, A. Lozano, S. Uclés, M.M. Gómez Ramos, A.R. Fernández-Alba*

Agrifood Campus of International Excellence (ceiA3), European Union Reference Laboratory for Pesticide Residues in Fruit & Vegetables, Department of Chemistry and Physics, University of Almeria, La Cañada de San Urbano, 04120 Almería, Spain

ARTICLE INFO

Article history:
Received 3 August 2015
Received in revised form
24 November 2015
Accepted 25 November 2015
Available online 28 November 2015

Keywords:
LC–MS/MS
GC–MS/MS
Pollen
Multiresidue pesticide analysis
Freezing-out
Zirconium dioxide

ABSTRACT

Several clean-up methods were evaluated for 253 pesticides in pollen samples concentrating on efficient clean-up and the highest number of pesticides satisfying the recovery and precision criteria. These were: (a) modified QuEChERS using dSPE with PSA + C18; (b) freeze-out prior to QuEChERS using dSPE with PSA + C18; (c) freeze-out prior to QuEChERS using dSPE with PSA + C18 + Z-Sep; and (d) freeze-out followed by QuEChERS using dSPE with PSA + C18 and SPE with Z-Sep. Determinations were made using LC–MS/MS and GC–MS/MS. The modified QuEChERS protocol applying a freeze-out followed by dSPE with PSA + C18 and SPE clean-up with Z-Sep was selected because it provided the highest number of pesticides with mean recoveries in the 70–120% range, as well as relative standard deviations (RSDs) typically below 20% (12.2% on average) and ensured much better removal of co-extracted matrix compounds of paramount importance in routine analysis. Limits of quantification at levels as low as 5 $\mu g\,kg^{-1}$ were obtained for the majority of the pesticides. The proposed methodology was applied to the analysis of 41 pollen bee samples from different areas in Spain. Pesticides considered potentially toxic to bees (DL50 < 2 μg/bee) were detected in some samples with concentrations up to 72.7 $\mu g\,kg^{-1}$, which could negatively affect honeybee health.

1. Introduction

Declines in bee colony numbers are being registered as much in the EU as in other parts of the world. Apart from the biodiversity implications, in economic terms, given that agriculture is such a key sector in the EU, the estimated value of pollination is around 22 € billion annually (of which 3292 € million is in Spain). Exposure to pesticides has been identified as one of the factors involved in bee Colony Collapse Disorder (CCD) [1–3]. When this event occurs, honeybee learning, memory, navigation and foraging activities are disrupted. These sublethal effects can be attributed to pesticides at concentration levels that are frequently encountered in contaminated pollen, nectar or guttation fluid [1,4] – found both by foraging honeybees and within the hive. In addition, the effects of many pesticides may be amplified by coexposure to other pesticides [3], such as the miticides used by beekeepers to combat epidemics and pesticides sprayed on crops.

To tackle the CCD problem, in December 2013, the European Commission adopted a proposal [5–7] to restrict the use of 3 pesticides belonging to the neonicotinoid family (clothianidin, imidacloprid and thiametoxam) for a two-year period. These restrictions were later followed by a ban on fipronil for seed treatment (EC Regulation N° 781/2013) [8]. At present, the Commission is reviewing current information taking relevant scientific and technical developments into account. In response to the need for contemporary knowledge regarding CCD, an analytical challenge has arisen to get accurate, effective and sensitive analytical methods to determine pesticide residues in matrices such as pollen samples, which are readily available in the environment where honey bees forage.

There are only a few large multiresidue methods described in the literature for the analysis of pesticide residues in pollen [4,9]. The most reported methodology has been developed to determine neonicotinoid insecticides [10–14]. However, honeybees are exposed to multiple pesticides via spray applications on crops near beehives and, in addition, beehives are treated with other pesticides such as miticides and fungicides to protect honeybees against parasites such as *Varroa* mites and *Nosema* fungus. It has been

* Corresponding author.
E-mail address: amadeo@ual.es (A.R. Fernández-Alba).

http://dx.doi.org/10.1016/j.chroma.2015.11.081
0021-9673/

documented that high levels of in-hive pesticides (at ppm levels) are co-occurring with lower but significant levels of other insecticides, fungicides and herbicides [4,9,15]. Consequently, a major challenge remains in finding simple and sensitive multiresidue approaches which encompass the large number of pesticides with a toxicity to honeybees that can contaminate pollen – this is especially the case due to the presence of many interfering compounds such as lipids (10–20%) and proteins (30–40%) present in this matrix. Accordingly, pollen sample pretreatment is more problematic given that the physico-chemical properties of the multi-analytes vary considerably thus making it difficult to remove interfering lipids and proteins without losing certain analytes.

Extracting pesticides from matrices containing high lipid and protein contents requires additional sample treatment strategies to completely remove these compounds prior to mass spectrometric detection in order to avoid strong ion suppression or enhancement and negative maintenance effects [4,16–18]. Recently, Chen et al. [10,11] have proposed a modified QuEChERS methodology for neonicotinoid analysis adjusted to pollen samples by adding a small fraction of hexane in acetonitrile to eliminate any lipids that interfere with the mass spectrometry. With respect to multiresidue analysis, Wiest et al. [4] developed a multiresidue method to quantify 80 contaminants, including pesticides, belonging to different chemical classes, in honey, honeybees and pollen. The addition of a very apolar solvent such as hexane in the extraction step proved efficient in removing coextractives from the pollen samples. However, despite the addition of hexane, detector saturation was observed at the same retention time as for procymidone and triadimenol, which explains their non-detection. A decrease in recovery was also observed for very apolar compounds.

The dSPE cleanup in QuEChERS methodology mainly involves primary secondary amine (PSA) and other alternatives such as octadecyl (C18), silica, aminopropyl (NH_2), strong anion exchange (SAX) and/or graphitized carbon black (GCB); however, the use of these sorbents either singularly or in combination does not ensure efficient extract purification for samples which contain elevated amounts of lipids and proteins [17–21].

Freezing-out in organic media is a simple method for lipid and protein removal from the extract requiring no reagent; instead, it involves only a straightforward operation and simple materials. This method has been used in combination with the QuEChERS methodology when further clean-up is necessary. Accordingly, efficient lipid and protein removal was achieved when freezing-out was applied to similar matrices such as beeswax [22], beebread [23] and honeybees [20].

The novel zirconia-based family of sorbents (SupelTM QuE Z-Sep), used both for QuEChERS (dSPE) and traditional cartridge SPE, represents an advance in QuEChERS technology for the clean-up of fatty matrices. The use of zirconia-based sorbents has proven successful for the QuEChERS clean-up of various fatty foods such as avocado [17,18], almonds [17,18] and edible vegetable oils [19] – when employed in pesticide residue analysis. Z-Sep consists of a mixture of C18 and silica coated with zirconium dioxide sorbents. The surface of zirconium dioxide includes Lewis acid, Brønsted acid and Brønsted base sites which, depending on the pH value, can retain carboxylic acids and hard Lewis bases such as R-SO^{3-}, R-$PO_3{}^-$ and R-COO^-. Thus, fatty acids, oleate anions, phospholipids as well as protein molecules with numerous carboxylic groups can be strongly adsorbed on the ZrO_2 surface [17,18]. Rajski et al. [17,18] evaluated two sorbents containing ZrO_2 (Z-Sep and Z-Sep$^+$) for pesticide analysis in avocado and almonds. The QuEChERS protocol with Z-Sep provided the lowest amount of coextracted matrix compounds and the highest number of pesticides with recoveries in the 70–120% range.

In the present work, we propose a simple, efficient and reliable technique based on a modified QuEChERS method, evaluating the efficiency of the clean-up step by comparison testing four different sample treatments: firstly, the addition of the dSPE sorbent combination (PSA + C18) in a single clean-up step; secondly, the inclusion of a freezing out step prior to dSPE with PSA + C18; thirdly, the addition of Z-Sep in dSPE; and finally, the addition of Z-Sep into a traditional SPE cartridge all followed by simultaneous liquid and gas chromatography-triple quadrupole-mass spectrometry (LC–MS/MS and GC–MS/MS) determination of 253 pesticides in pollen samples.

To compare the amount of matrix compounds present in the final extracts from the different clean-up pretreatments tested, a LC-QTOF was used prior to determination by LC–MS/MS and GC–MS/MS. The objective of using the LC-QTOF system was to select the most effective cleaning procedure, crucial in avoiding problems of ionization efficiency and in the detection systems of the analytical instruments. Additionally, a higher degree of specificity was obtained thus minimizing the appearance of false positives and/or false negatives.

The validated method was employed in a survey carried out on 41 real samples from apiaries located in different regions of Spain.

2. Experimental

2.1. Reagents and materials

All pesticide standards of high purity were obtained from Dr. Ehrenstorfer (Augsburg, Germany) and Riedel-de Haën (Selze, Germany) and were stored at −30 °C. Individual pesticide stock solutions (1000–2000 mg L^{-1}) were prepared in acetonitrile and ethyl acetate and were stored in amber screw-capped glass vials in the dark at −20 °C. Individual standard solutions for optimization and two standard-mix solutions for calibration were prepared from the stock standards.

Ultra-gradient HPLC-grade acetonitrile was obtained from Sigma–Aldrich (Steinheim, Germany). Trisodium citrate dihydrate was purchased from Fluka (Steinheim, Germany). Sodium chloride was purchased from J.T. Baker (Deventer, The Netherlands). Disodium hydrogencitrate sesquihydrate was obtained from Sigma–Aldrich (Steinheim, Germany). Anhydrous magnesium sulphate was supplied by Panreac (Barcelona, Spain). C18 was purchased from Agilent Technologies (Santa Clara, CA, USA). PSA and Z-Sep were obtained from Supelco (Bellefonte, PA). A Milli-Q-Plus ultra-pure water system from Milli-pore (Milford, MA, USA) was used throughout the study to obtain the HPLC-grade water used during the analyses and to hydrate the pollen. Formic acid (98% purity) was purchased from Fluka (Buchs, Switzerland). Dry ice was supplied from technical services (University of Almería).

2.2. Equipment

For LC analysis, we used an Agilent 1290 HPLC system (Agilent Technologies, Palo Alto, CA, USA) with a binary pump. It was equipped with a reversed-phase C8 analytical column of 2.1 mm × 100 mm and 1.8 μm particle size (Agilent Zorbax Eclipse Plus). Compounds were separated using acetonitrile with 0.1% formic acid and 5% MilliQ water (mobile phase A) and MilliQ water with 0.1% formic acid (mobile phase B). The flow rate was kept constant at 0.3 mL min^{-1} and the gradient programme was set as follows: 20% A (initial conditions) was kept constant for 2 min followed by a linear gradient up to 100% A in 13 min; after which the mobile phase composition was maintained at 100% A for 2 min. The re-equilibration time was 2.5 min. The injection volume was 10 μL. For the mass spectrometric analysis, a 6490 QqQ MS/MS system (Agilent Technologies, Palo Alto, CA, USA) equipped with an electrospray ionization source (ESI) operating in positive ionization mode