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Presenting Author

Surname and name Bergomi Andrea		
Company or Organization University of Milan		
Address Via Golgi 19		*Zip code – Town – Country* 20133
Telephone 334769456	*Fax*	*E-mail* andrea.bergomi@unimi.it

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Title Application of a QuEChERS LC-MS/MS method for the determination of pesticides in environmental bioindicators: *Apis mellifera* and honey.
Authors Andrea Bergomi, Alberto Zaglio, Valeria Comite, Vittoria Guglielmi, Marcello Freguglia, Monica Bornatici, Giuseppina Amato, Marco Rizzi, Giacomo Notaro, Sonia Vitaliti, Maurizio Barbieri, Paola Fermo.

Company(es) or Organization(s)

University of Milan

ATS-Milan Laboratorio di Prevenzione

ATS-Milan Dipartimento Veterinario

Application of a QuEChERS LC-MS/MS method for the determination of pesticides in environmental bioindicators: *Apis mellifera* and honey.

Andrea Bergomi[1], Alberto Zaglio[1], Valeria Comite[1], Vittoria Gugliemi[1], Marcello Feruglia[2], Monica Bornatici[2], Giuseppina Amato[2], Marco Rizzi[2], Giacomo Notaro[2], Sonia Vitaliti[2], Maurizio Barbieri[3], Paola Fermo[1]

[1] University of Milan, Department of Chemistry, Via Golgi 19, 20133, Milan, Italy
[2] ATS-Milan Laboratorio di prevenzione, Via Juvara 22, 20129, Milan, Italy
[3] ATS-Milan Dipartimento Veterinario, Via Juvara 22, 20129, Milan, Italy

Summary: *The present study presents the application of a multi-residual analysis method for the determination of 165 pesticides, both EU-approved and non-EU-approved, in 101 bee samples and 27 honey samples collected over a five-month period (June-October) in 36 different beehives located in nine areas surrounding the metropolitan city of Milan. The analytical determination has been carried out by LC-MS/MS technique following a QuEChERS extraction treatment.*

Keywords: *pesticides, LC-MS/MS, bees*

1. Introduction

Pollinators such as honey bees (*Apis mellifera*) possess numerous qualities which make them good bio-indicators, including the ability to accumulate the analytes of interest and being easy to identify and to sample [1]. They are therefore ideal for monitoring environmental stressors and obtaining information about the quality of the environment in which they live in [2]. The main advantage of bees as bio-indicators, which is a direct consequence of their activity as pollinators, is their close connection with the territory and the environment [3]. In fact, they can come into contact with pesticides through various means of exposure, the most important of which are: the collection of pollen and nectar from previously polluted flowers and the accumulation of airborne substances during flight [4]. Thanks to the interaction with the bees, pesticides are then able to enter the hive where they are distributed in all its compartments, such as wax and honey [5].

This paper presents the application of a multi-residual analysis method for the determination of 165 pesticides, both EU-approved and non-EU-approved, in 101 bee samples and 27 honey samples collected over a five-month period (June-October) in 36 different beehives located in nine areas surrounding the metropolitan city of Milan. The method is based on the LC-MS/MS technique after QuEChERS extraction treatment, suitably optimised to be adapted to the matrices under consideration.

2. Materials and Methods

The optimised method of analysis, in accordance with current regulations on pesticide analysis, was, prior to its application to the biomonitoring samples, validated following the indications contained in the SANTE guideline [6]. The biomonitoring phase was then carried out. A total of 36 beehives located in nine different areas surrounding the metropolitan city of Milan were sampled. From each one, around 1 g of bee (13-15 individuals) and 5 g of honey produced by the same bees were taken monthly, from June to October.

The analytes of interest were extracted from the matrices using the QuEChERS technique, which is divided in two steps. The first is a solid-liquid extraction performed using an organic solvent (acetonitrile) followed by its separation from the aqueous phase. The second phase involves the purification of the extract through a d-SPE (dispersive-Solid Phase Extraction) procedure in which an insoluble salt is dispersed within the mixture to retain interferents and impurities without affecting the analytes of interest. The extract obtained from this procedure then underwent LC-MS/MS analysis.

The instrument used for the identification and quantification of the analytes was a Shimadzu ExionLC HPLC with a Qtrap Sciex 6500 mass detector. An ESI Ion Drive turbo V Sciex with both positive and negative ionisation modes was used as the ionization source, operating at a temperature of 350°C and Ion Spray Voltage (IS) 3500(V) and -3500(V) respectively for the positive and negative ionisation modes.

A C18 column (2.1×100 mm, 1.8µm) was used as the stationary phase, while ammonium formate 10 mM in H_2O at 0.1 % (v/v) formic acid (mobile phase A) and ammonium formate 10 mM in MeOH at 0.1 % (v/v) formic acid (mobile phase B) were used as the two mobile phases with a gradient elution for the entire 15 minutes of the chromatographic run, as reported in Table 1. Moreover, a column temperature of 50°C and a flow rate of 0.450 mL/min were used.

Table 1. Gradient elution details

Time / min.	Mobile phase A / %	Mobile phase B / %
0.00	95.0	5.0
0.20	95.0	5.0
11.00	0.0	100.0
12.25	0.0	100.0
12.56	5.0	95.0
15.00	5.0	95.0

The mass spectrometer and detector operated in MRM mode using two transitions: Q1 (precursor ion) and Q2 (product ion) with an MRM Detection Window of 90 seconds. Identification of each analyte was performed based on retention time and ion ratio using the two transitions, while quantification was performed by integrating the peak of the transition that gave the greatest instrumental response.

3. Results and Discussion

The results obtained following LC-MS/MS do not indicate the presence of particular trends both in terms of geographical location and sampling periods. With regards to the quantification of the analytes, only 17 pesticides were detected above the limit of detection (LOD) and, of these, only 6 above the limit of quantification (LOQ). The species in question were: tebufenpyrad, cypermethrin, imidacloprid, thiabendazole, hexaconazole, diflubenzuron, buprimate, diazinon, difenoconazole, dimethomorph, fenazaquin, fluopicolide, metalaxyl, metrafenone, piperonyl butoxide, prosulfocarb and triflumuron. Their presence may be attributable to several factors, such as the sampling period, the type of sampling areas and the type of active substances detected.

Within the detected species, cypermethrin was the most abundant and widespread, present in almost all the sites and during the entire sampling period. This species is an active ingredient contained in both household and livestock products for the treatment of flies and mosquitoes. The maximum concentration found for cypermethrin was 0.013 ± 0.007 mg kg^{-1}, which is below the maximum residue levels indicated by the European Commission regulation for fruits, vegetables and products of animal origin [7].

The results show an overall acceptable presence of pesticides in the samples analyzed, without highlighting any alarming contamination cases.

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