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Acetonitrile extraction and dual-layer solid phase extraction clean-up for pesticide residue analysis in propolis

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ABSTRACT

Propolis is a very complex mixture of substances that is produced by honey bees and is known to be a rather challenging matrix for residue analysis. Besides resins, flavonoids and phenols, high amount of wax is co-extracted resulting in immense matrix effects. Therefore a suitable clean-up is crucial and indispensable. In this study, a reliable solid phase extraction (SPE) clean-up was developed for pesticide residue analysis in propolis. The clean-up success was quickly and easily monitored by high-performance thin-layer chromatography with different detection possibilities. The final method consists of the extraction of propolis with acetonitrile according to the QuEChERS method followed by an effective extract purification on dual-layer SPE cartridges with spherical hydrophobic polystyrene-divinylbenzene resin/primary secondary amine as sorbent and a mixture of toluene/acetone (95:5, v/v) for elution. Besides fat-soluble components like waxes, flavonoids, and terpenoids, more polar compounds like organic acids, fatty acids, sugars and anthocyanins were also removed to large extent. Method performance was assessed by recovery experiments at spiking levels of 0.5 and 1 mg/kg (n = 5) for fourteen pesticides that are relevant for propolis. Mean recoveries determined by HPLC-MS against solvent standards were between 40 and 101%, while calculation against matrix-matched standards provided recoveries of 79–104%. Precision of recovery, assessed by relative standard deviations, were below 9%. Thus, the developed dual-layer SPE clean-up enables the reliable pesticide residue analysis in propolis and provides a suitable alternative to time-consuming clean-up procedures proposed in literature.

1. Introduction

Propolis is a brownish resinous material collected by honeybees from different leaf buds, plants and exudates [1–3]. Bees use propolis as coating material for hive parts, to seal crevices and cracks in the hive and to repair combs [2,4]. The composition of propolis is directly related to the composition of the collected bud exudates and, therefore, constituents can vary widely depending on the botanical and geographical origin and, additionally, on the time of collection [1,5]. Propolis originating from the temperate zone, including Europe, Asia, and North America, is characterized by a relatively similar composition [4]. The chemical composition of propolis turned out to be a very complex mixture of at least 300 organic substances that have been identified in different propolis samples [4]. The main constituents are phenolic compounds like flavonoid aglycones, aromatic acids and their esters, constituting more than 50% the mass of propolis [6]. Due to its special chemical composition, mainly natural antioxidants (phenolic compounds), propolis has biological and pharmacological properties, and has been intensively used for a long time as a popular remedy in folk medicine [7–9]. Recently, propolis is also added to food, beverages, and also to cosmetic products, sold as "biocosmetic" and "health food", claiming beneficial health effects mainly in the area of inflammation, heart disease, diabetes, and even cancer [1,2,4,6,7,9–11].

Inevitably, honey bees get in contact with pesticides during the collection of pollen and leaf buds, and upon contact with crops. Bees accumulate pesticides and transport them back to the beehive, where the entire bee swarm absorbs them and eventually contaminate their own products such as propolis. In the European Union (EU) maximum residue limits (MRLs) have been set by the directive 91/414/EEC [12], harmonized and implemented by the regulation 2005/396/EC [13] on maximum residue levels of pesticides in or on food and feed of plant and animal origin for over 450 pesticides. However, pesticides residues in propolis are not explicitly mentioned, but there exist maximum residue levels for honey and other apiculture products, where propolis is part of. Furthermore, propolis is frequently used as food supplement. In the EU, food supplements are regulated as foods, but the legislation mainly

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focuses on vitamins and minerals used as ingredients of food supplements and not on pesticide residues. When propolis is processed in cosmetics like ointments and creams, regulation 2009/1223/EC [14] needs to be applied, while only some pesticides are regulated. Anyway monitoring of pesticide residues in propolis becomes more and more important given the increasing usage of propolis in recent years as a consequence of its image as a healthy, innocuous and natural product [3,4,7,8,15]. Furthermore, due to the massive cases of bees' death in recent years, there has been an increasing apiculture concern related to the presence of pesticide residues in propolis. Consequently, reliable and robust analytical methods for pesticide residue analysis in propolis are required.

Generally, pesticide residue analysis requires a sequence of steps. First, the extraction of pesticides with organic solvents from the matrix, followed by the most important clean-up step and the final high-performance liquid or gas chromatographic (HPLC or GC) determination, commonly by mass spectrometry (MS). Co-extracted matrix components from complex matrices are responsible for the well-known matrix effects in pesticide residue analysis by HPLC-MS and GC-MS [16,17]. Co-eluting compounds may be the source of false negatives, false positives, or inaccurate quantification, depending on the target analytes and the matrix [17]. Precision and accuracy are indispensable for pesticide residue analysis methods and directly correlate with the removal of interfering compounds causing matrix effects [16–20]. Consequently, sample extraction and clean-up are the most crucial steps in residue analysis. This is especially true for the difficult (dirty) propolis matrix. The big challenge is the extraction and separation of the relatively lipophilic pesticides from the hydrophobic resinous propolis matrix (about 50% resins and 30% wax [1,3]). Additionally, huge amounts of further organic compounds are co-extracted, which also have to be separated from pesticides during clean-up. In this context it is not surprising that determination of pesticide residues in propolis is rather seldom reported and methods in literature are scarce, whereas, in contrast many methods for pesticide residue analysis in other bee products such as honey are accessible, e.g. reviewed by Fernandez et al. [21]. The only methods made publicly available for pesticide residue analysis in propolis describe common extraction methods and mostly time-consuming clean-up methods. Liquid–liquid extraction (LLE) was performed with several single solvents and solvent mixtures (hexane, ethyl acetate, methylene chloride, and acetone) and was assisted by sonication, shaking and homogenization [5,21–23]. Orsi et al. [24] described a method based on accelerated solvent extraction with ethyl acetate. Additionally, some matrix solid-phase dispersion methods are reported in literature [15,23,25–27]. Different dispersive materials (celite, silica, octadecyl silica (C18), $Al_2(SO_4)_3$) and several single solvents and binary solvent mixtures (methylene chloride, acetone, acetonitrile, and ethyl acetate) were proposed for extraction. For clean-up, only very few methods are described in literature. Gel permeation chromatography (GPC) is typically applied for complex matrices from animal or botanical origin with high fat content. This rather time and solvent consuming approach is suggested for clean-up of propolis extracts by Orsi et al. [24] and Pareja et al. [23]. Solid phase extraction (SPE) is described by Chen et al., who worked with tandem graphitized carbon and florisil cartridges [5]. Determination is often performed by GC coupled to electron capture detection [5,23,24,28] or MS [15,23,25,27], less frequently by HPLC-MS [23], depending on the spectrum of pesticides analyzed. However, among the general deficiencies of the reported methods for clean-up of propolis extracts, there is either an insufficient purification leading to matrix effects or the need for time-consuming procedures with high solvent consumption [5,15,22–24,26,27].

Therefore, the aim of the present study was to develop an effective extraction and clean-up for the determination of pesticide residues in propolis. Different solvents for extraction and clean-up methods were to be evaluated and adapted to the specific characteristics of the propolis matrix and the pesticides. High-performance thin-layer chromatography (HPTLC) allowed a fast and easy control of the clean-up success. HPLC-MS analysis in the SIM mode was performed to determine recoveries of pesticides.

2. Material and methods

2.1. Chemicals and materials

Acrinathrin (99.0%), azoxystrobin (99.0%), boscalid (99.0%), chlorfenvinphos (94.5%), coumaphos (99.0%), diethyltoluamide (DEET) (98.0%), dimoxystrobin (99.0%), flumethrin (96.8%), iprodione (98.0%), tau-fluvalinate (94.0%), terbuthylazine (98.5%), thiacloprid (99.2%) were purchased from Ehrenstorfer (Augsburg, Germany). Chlorpyrifos (99.9%), and pirimicarb (99.0%) were obtained from Sigma-Aldrich (Steinheim, Germany). The internal standard tris(1,3-dichloro-2-propyl)phosphate (TDCPP) (97.5%) was purchased from High Purity Compounds (Cunnersdorf, Germany). Primuline (dye content 50%) was purchased from Sigma-Aldrich. Sodium chloride (pro analysis) and di-sodium hydrogencitrate 1.5-hydrate (>99%) were purchased from Merck (Darmstadt, Germany), and sodium citrate tribasic dihydrate (>99%), magnesium sulphate, anhydrous (reagent grade, ≥97%) and ammonium formate (for mass spectrometry, ~99.0%) from Sigma-Aldrich. Acetonitrile, methanol (both LC–MS, Chromasolv), and toluene (for pesticide residue analysis) were obtained from Sigma-Aldrich. Acetone (Rotisolv pestilyse) was obtained from Carl Roth (Karlsruhe, Germany). Ultrapure water (>18 MΩ cm) was supplied by a Synergy System (Millipore, Schwalbach, Germany). Polyethylene (PE) frits (20 μm porosity) were purchased from Sigma-Aldrich. Bondesil–PSA (primary secondary amine, 40 μm), was obtained from Varian (Palo Alto, USA). Chromabond HR-X polypropylene columns (hydrophobic polystyrene-divinylbenzene adsorbent resin, 6 mL, 200 mg, 85 μm) were purchased from Machery-Nagel (Düren, Germany). TLC aluminum foil silica gel 60 NH_2 F_{254}s, 20 cm × 20 cm from Merck were prewashed with acetonitrile, dried in fume-hood for 30 min, and were stored in a SICCO Star-Vitrum desiccator (Bohlender, Grünsfeld, Germany) until use to prevent contamination.

2.2. Samples

Propolis samples from different origins were obtained from the Apicultural State Institute, University of Hohenheim (Stuttgart, Germany). Samples were frozen for 2 h at −20 °C and finely milled in a Tube Mill control (IKA, Staufen, Germany) for 30 s at 12,000 min^{-1} before analysis. A mixture of the different propolis samples was prepared for evaluation of different extraction and clean-up methods regarding the removal of co-extracted matrix.

2.3. Extraction and clean-up procedures

Several methods were tested for extraction and clean-up. Propolis extracts were prepared according to the QuEChERS method [29], the ChemElut method [30] and the Swedish ethyl acetate method [31,32], all extracts with a concentration of 0.2 g sample/mL. QuEChERS extracts were subjected to different clean-up strategies including LLE according to Cajka et al. [33], SPE, and dispersive SPE (dSPE) according to the QuEChERS method [29]. For evaluation of the dSPE and SPE approaches, an aliquot of the QuEChERS extract of 2 mL and 250 μL, respectively, was used in all cases, and several sorbents and elution solvents were tested. Extracts after dSPE were analyzed by HPTLC without further treatment. Eluates after SPE were evaporated under a gentle stream of