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Determination of phenolic compounds in honey using dispersive liquid–liquid microextraction

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ABSTRACT

Honey is a valuable functional food rich in phenolic compounds with a broad spectrum of biological activities. Analysis of the phenolic compounds in honey is a very promising tool for the quality control, the authentication and characterization of botanical origin, and the nutraceutical research. This work describes a novel approach for the rapid analysis of five phenolic acids and 10 flavonoids in honey. Phenolic compounds were rapidly extracted and concentrated from diluted honey by dispersive liquid–liquid microextraction (DLLME) and then analyzed using high performance liquid chromatography with UV absorbance detection (HPLC–UV). Some important parameters, such as the nature and volume of extraction and dispersive solvents, pH and salt effect were carefully investigated and optimized to achieve the best extraction efficiency. Under the optimal conditions, an exhaustive extraction for twelve of the investigated analytes (recoveries >70%), with a precision (RSD < 10%) highly acceptable for complex matrices, and detection and quantification limits at ppb levels (1.4–12 and 4.7–40 ng g^{-1}, respectively) were attained. The proposed method, compared with the most widely used method in the analysis of phenolic compounds in honey, provided similar or higher extraction efficiency, except in the case of the most hydrophilic phenolic acids. The capability of DLLME to the extraction of other honey phytochemicals, such as abscisic acid, was also demonstrated. The main advantages of developed method are the simplicity of operation, the rapidity to achieve a very high sample throughput and low cost.

1. Introduction

Honey is a natural sweetener used since the earliest times as a sugar substitute, an ingredient or a natural preservative in many of manufactured foods. Moreover, it has been used in many cultures for its medicinal properties and it has been reported to be effective in gastrointestinal disorders, in the healing of wounds and burns and as antimicrobial agent [1,2]. Nowaday, honey is used in apitherapy for treating certain conditions and diseases as well as promoting overall health and well-being [1].

Honey is similar in antioxidant capacity to many fruits and vegetables on a fresh-weight basis [3] and it has been hypothesized a potential therapeutic role of honey in treating cardiovascular diseases mainly due to the flavonoid-mediated activities. The consumption of honey has been shown to inhibit the oxidation of low density protein, vasodialate blood vessels due to nitric oxide production, decrease platelet aggregation, and exert analgesic and anti-atherogenic effects: each of which may decrease cardiovascular risk [4]. These beneficial effects in one or more physiological functions highlight the role of honey as a valuable functional food [1].

The proprieties of honey are usually related to its minor constituents (polyphenols and other phytochemicals, enzymes, ascorbic acid, Maillard reaction products, carotenoid-like substances, organic acids and amino acids and proteins), especially flavonoids and phenolic acids [2,5].

Honey polyphenols originate from the plants used to collect the nectar and by the contact with propolis in the hive [2,5]. The main phytochemicals reported in honey are flavonoids (apigenin, chrysin, galangin, hesperetin, kaempferol, luteolin, myricetin, pinobanksin, pinocembrin, quercetin, and tricetin), phenolic acids (caffeic, chlorogenic, coumaric, ellagic, ferulic, gallic, homogentisic, phenyllactic, protocatecuic, syringic and vanillic acids) and their derivatives [1,5].

Many studies show that honey, depending on the floral source and the geographical origin, possesses different phenolic profiles and antioxidant, antibacterial, or radical-scavenging activities [5].

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Therefore, the analysis of phenolic compounds is a useful analytical tool to evaluate the quality of honey, which is judged by its botanical origin and nutraceutical value.

Generally, the analysis of phenolic compounds in honey involves the elimination of matrix components, mainly sugars, and the preconcentration of analytes before the determination, which is carried out most often by HPLC [1,2]. Liquid–liquid extraction (LLE) with organic solvents and solid phase extraction (SPE) are frequently used to extract and concentrate the phenolic compounds from honey [1,2]. SPE on Amberlite XAD-2 followed by LLE with diethyl ether [6] is the most popular technique applied, but the use of C18 sorbents in SPE clean-up has also been reported [7–9]. These methods are expensive, time consuming and use large amounts of solvents. The availability of fast and inexpensive analytical procedures for the determination of phenolic compounds in honey is highly demanded for the quality control, the nutraceutical research and the authentication and characterization of botanical origin. In this regard, advanced extraction techniques have demonstrated superior performance over conventional methods [2,10,11].

Dispersive liquid–liquid microextraction (DLLME) is a newly extraction technique with numerous advantages such as operational simplicity, low cost, modest consumption of organic solvents, which reduces its environmental impact, short extraction times, high recoveries and significant enrichment factors [12]. DLLME is based on the complete dispersion of an extractant solvent into an aqueous sample with the help of a disperser solvent that is miscible within both components and causes the formation of a cloudy solution. The main purpose is to enlarge the contact area between extractant and sample in a simple manner in order to expedite mass transfer with reduced volumes of sample and solvents [12]. DLLME has been used mainly in the analysis of environmental water matrices with excellent analytical performance for different types of analytes [13,14]. A few DLLME applications have been devoted to the determination of organic compounds in highly complex matrices, such as food [15–17]. However, the complexity of food samples makes its application in this area more problematic due to the presence of potential matrix interferences [18]. Regarding the honey, recently DLLME has been applied in the analysis of pesticides and antibiotics [19–22].

The aim of this research was to develop a fast and inexpensive DLLME method suitable to the determination of phenolic compounds in honey. Among the main phytochemicals reported in honey, five phenolic acids (caffeic acid, ferulic acid, *p*-coumaric acid, syringic acid and vanillic acid) and 10 flavonoids (apigenin, chrysin, galangin, hesperetin, kaempferol, luteolin, myricetin, pinobanksin, pinocembrin and quercetin) were selected as target analytes. The experimental parameters affecting on the DLLME efficiency were carefully studied and optimized. HPLC–UV was selected as detection method and HPLC coupled to high-resolution mass spectrometry (HPLC–HRMS) was used to characterize the compounds extracted by DLLME and to investigate the applicability of this extraction technique to other phytochemicals of honey. Finally, the developed analytical procedure was applied to the analysis of honey samples from Calabria region (Italy) and the analytical performance were compared with the method most widely used in the analysis of phenolic compounds in honey (SPE with Amberlite XAD-2 followed by LLE with diethyl ether [23]).

2. Experimental

2.1. Standards and materials

Analytical-grade acetone (Me_2CO), acetonitrile (MeCN), chlorobenzene (C_6H_5Cl), chloroform ($CHCl_3$), dichloromethane (CH_2Cl_2), dimethyl sulfoxide (DMSO), ethanol (EtOH) and methanol (MeOH) were obtained from Merck (Germany). HPLC-MS-grade MeOH and water were purchased from Romil (Cambridge, UK). Amberlite XAD-2 resin (pore size 9 nm; particle size 0.3–1.2 mm, Supelco), ammonium sulfate $(NH_4)_2SO_4$, formic acid (HCOOH), fructose, glucose, hydrochloric acid (HCl) and sodium chloride (NaCl) were provided by Sigma-Aldrich (Milan, Italy). Regenerated cellulose syringe filters (CHROMAFIL Xtra RC-45/25, pore size 0.45 μm, diameter 25 mm, Macherey-Nagel) were purchased from Delchimica (Naples, Italy).

Standards of (±)-*cis*,*trans*-abscisic acid (*c*,*t*-ABA), apigenin (API), caffeic acid (CAA), chrysin (CHR), ferulic acid (FEA), hesperetin (HES), pinobanksin (PIB), *p*-coumaric acid (*p*-COA), quercetin (QUE), syringic acid (SYA), vanillic acid (VAA), were obtained from Sigma-Aldrich. Galangin (GAL), kaempferol (KAE), luteolin (LUT) myricetin (MYR) and pinocembrin (PIC) were purchased from Extrasynthese (Lyon, France). Standard stock solutions (1 mg mL^{-1}) of each compound were prepared in methanol and stored at 4 °C. Diluted solutions and mixtures were made in MeOH/H_2O 1:4, v/v.

2.2. Samples

Seven honey samples of different botanical origin were directly received from different beekeepers. The samples were collected in Calabria (Italy) during 2012 and were stored at 4 °C in the dark. The Calabrian honey (CH) samples were: CH1–CH3 acacia (*Robinia pseudoacacia* L.), CH4–5 sulla (*Hedysarum coronarium*), CH6 thistle and CH7 citrus honeys.

An artificial honey (supersaturated solution of 80% sugar), reflecting the main components of honey, was prepared dissolving 45 g of F and 35 g of G in distilled water up to final weight of 100 g. This solution was spiked with target compounds at the 1 μg g^{-1} level and used as free analyte matrix in the DLLME optimization and validation studies.

2.3. Dispersive liquid–liquid microextraction

Fortified artificial honey and honey samples were homogenized by manual stirring (3 min) and a representative 10 g aliquot was dissolved with acidified water (pH 2) to the final volume of 100 mL to obtain a 10% (w/v) solution. Diluted solution was filtered through a 0.45 μm syringe cellulose filters to remove solid particles. Successively, 10 mL aliquot of diluted honey sample, or artificial honey, was placed into a 15 mL conical tube. Under final DLLME conditions, 3 g of $(NH_4)_2SO_4$ were added and the pH of aqueous solution was adjusted at 2 with HCl 1 N. Then, 450 μL $CHCl_3$ (DLLME extractant) and 750 μL Me_2CO (DLLME disperser) were added in turn to the aqueous solution. The ternary component system was vigorously shaken by hand for 10 s and a stable cloudy solution was formed. Successively, the mixture was centrifuged for 5 min at 6000 rpm (ALC centrifuge PK 120, Thermo Electron Corporation) to separate two phases. The settled phase was quantitatively transferred to a 2 mL eppendorf vial and dried under a gentle nitrogen flow. Finally the residue was reconstituted with 100 μL of MeOH/H_2O 1:4, v/v, before the chromatographic analysis.

2.4. Solid phase extraction with XAD-2

SPE was carried out as described previously by Ferreres [23]. Briefly, 5 g of Amberlite XAD-2 resin were soaked in methanol, stirred for 10 min and then packed into a 12 mL SPE cartridge (5 × 1.5 cm). Honey samples or artificial honey (10 g) were mixed with 50 mL of distilled water and adjusted to pH 2 with HCl 1 N. The solution was filtered through a 0.45 μm syringe cellulose filters and loaded on preconditioned (with acidified water, pH 2)