PAPER

PHYTOCHEMICAL COMPOSITION AND ANTIOXIDANT ACTIVITY OF TUSCAN BEE POLLEN OF DIFFERENT BOTANIC ORIGINS

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ABSTRACT

Within the apicultural products, the honey bee-pollen is growing in commercial interest due to its high nutritional properties. For the first time, bee-pollen samples from Tuscany (Italy) were studied to evaluate botanical origin, phytochemical composition and antioxidant activity. The investigated pollen loads were composed of three botanical families: *Castanea*, *Rubus* and *Cistus*. The highest levels of proteins and lipids were detected in *Rubus* pollen. *Castanea* pollen contained greater polyphenols, flavonoids and anthocyanins content, while the highest flavonols level was detected in *Cistus* pollen. These results were also confirmed by front-face fluorescence spectroscopy, used here, for the first time, as a fast tool to characterize bee-pollens.

- Keywords: antioxidant activity, pollen, bioactive compounds, chemical composition, fluorescence, Tuscany -

1. INTRODUCTION

For centuries the apicultural products have been used in phytotherapy as well as in diet for their health positive implications (KROYER and HEGEDUS, 2001; FERRERES *et al.*, 2010; ABOUDA *et al.*, 2011).

Bee-gathered pollen (bee-pollen) is an apicultural product of great commercial interest owing to its high nutritional value and physiological properties representing an important source of energy and proteins for human nutrition (ABOUDA *et al.*, 2011).

Worker-bees gather pollen from flowers and, after processing it with some proteic gland-secretion, they package it on their curbicula forming pollen loads before returning to the hive (PINZAUTI *et al.*, 2002; SCARSELLI *et al.*, 2005). Generally, a single pollen load is a one colored little pellet reflecting a homogeneous and monospecific pollen content. Bee-pollen loads are stilled from the worker bees at their entrance to the hive by special pollen loads traps. In accordance to the season, to the timing of collection by the beekeepers and to the post collection management is possible to obtain mono- or poly-pollen species loads. Amounts of pollen loads ready to be commercialized and consumed by human and animals is called bee-pollen. So it is possible to find bee-pollen of one specific flower (monoflora) or belonging to several flower species (polyflora), as well is possible to blend different monofloral in order to create mixtures of bee-pollen with characteristic organoleptic properties and quality attributes.

Nowadays, bee-pollen represents the richest and most complete natural food supplying high levels of carbohydrates (13-55%), proteins (10-40%), particularly free aminoacids, enzymes, cofactors, lipids (1-13%), including fatty acids and sterols, minerals, trace elements and vitamins, especially B group, A, C and E. Fresh and dry bee-pollen loads hold a different water content, ranging from 20-30% in the original form and 4-10% if dried, affecting organoleptic and "shelf-life time" properties (CAMPOS *et al.*, 2008; PASCOAL *et al.*, 2014).

Moreover, it is also an excellent source of bioactive compounds, such as phytosterols, carotenoids and polyphenols (especially flavonoids), that exert antioxidant, anti-inflammatory, antimicrobial, anti-allergic and antitumoral effects (MORAIS *et al.*, 2011; PÉREZ- PÉREZ *et al.*, 2012; FRATINI *et al.*, 2014). Very recently, several research works were published showing that bee products, such as propolis and pollens, possess a sedative effect and may be effective in protecting humans against depression and similar diseases (YILDIZ *et al.* 2014); additionally, preliminary studies show that pollens have a hepatoprotective potential (YILDIZ *et al.* 2013). Pollens effects on improving immune, cardiovascular and digestive systems as well as their therapeutic effects have been mainly related to the polyphenol content and chemical composition (PASCOAL *et al.*, 2014). In particular, the phenolic profile of bee-pollen consists of flavonol, glycosides and aglycones, and hydroxycinnamic acids, that can be present in free forms or combined with other pollen components (CHANTARUDEE *et al.*, 2012; FANALI *et al.*, 2013). However, as well as chemical composition, the phytochemical profile is affected by soil type, beekeeping management, climatic and preservation conditions, and especially by botanical origin (ALMEIDA-MURADIAN *et al.*, 2005; ARRUDA *et al.*, 2013; CAMPOS *et al.*, 2008). Aromatic aminoacids, many polyphenols, some enzyme cofactors, but also some water- or lipid-soluble vitamins and pigment's derivatives contained in bee-pollen are fluorescent intrinsic compounds (JØRGENSEN *et al.*, 1992; LAKOWICZ, 2006).

Front-face (FF) fluorescence spectroscopy is a non-destructive, rapid and sensitive technique suitable for complex and opaque samples, otherwise traditional right-angle florescence (KAROUI *et al.*, 2007; ZANDOMENEGHI *et al.*, 2005). In particular, this technique has been proved to be very effective in studying powders, crystalline or amorphous samples, and complex matrix, such as food (ZANDOMENEGHI, 1999; ZANDOMENEGHI and ZANDOMENEGHI, 2009; AIRADO-RODRÍGUEZ *et al.*, 2011; KULMYRZAEV *et al.* 2005). In this context, FF fluorescence could be a useful instrument to obtain a fingerprint of different bee-pollen types, advantageous to study and compare them.

In this study, we examined the botanical origin, the chemical composition (moisture, proteins, carbohydrates, lipids, ash) and the antioxidant profile (the total polyphenols and the flavonoids, flavonols and anthocyanins subclasses) of different color fractions of an organic Tuscan (Italian) bee-pollen sample. Moreover, we proposed for the first time a rapid qualitative evaluation of emission, excitation and synchronous spectra, obtained by front-face spectroscopy, of bulk state and ethanolic extracts of the different pollen types showing the main classes of identified fluorescent molecules. Lastly, to evaluate the synergic effect of the bee-pollen bioactive components we analyzed by DPPH and ORAC assay the free radical scavenging activity and the antioxidant capacity of both separate and mixed color fractions.

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

All standards and reagents were of analytical grade. Absolute ethanol, methanol, hydrochloric acid, trichloroacetic acid, diethyl ether, sodium carbonate, sodium idrosside, potassium chloride, sodium acetate, Folin-Ciocalteu rea-

gent, catechin, gallic acid, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 1,1-diphenyl-2-picrylhydrazyl (DPPH) and fluorescein sodium salt were purchased from Fluka-Sigma-Aldrich, Inc. (St. Louis, MO), as well as the solid standards used in spectroscopic analysis tryptophan, b-carotene, gallic acid, 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, vanillic acid, caffeic acid, ferulic acid, p-coumaric acid, quercetin dihydrate and the vitamins C, B3, B2, B6 and B9. Hydroxide peroxide, sulfuric acid, boric acid and Kjeldahl tablets were purchased from Merck (Readington, NJ). Sodium nitrite and aluminum chloride were purchased from Carlo Erba (Milan, IT), while 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) was purchased from Polysciences, Inc. (Warrington, PA).

2.2 Plant materials

Bee-pollen resulted from the blend of pollen loads collected during sunny days by the beekeeper using 10 beehives equipped with bottom-fitted pollen traps located in Massa Macinaia (Latitude 43.80638- Longitude 10.54213) in Lucca Province (Tuscany, Italy) between April and July 2013. In total 1 kg of bee-pollen was collected. The blended fresh bee-pollen was stored at -20°C in the dark until further analysis.

2.3 Palynological analysis

Within the bee-pollen sample the pollen loads were divided by colour into three groups (Fig. 1). Twenty single pollen loads of each colour were analyzed by microscope. Each single pollen load prepared by washing the pollen with distilled water and using glycerin jelly for permanent preparations. Pollen grains identification was performed by optical microscope with total magnification (400X and 1000X).

A reference collection of Pisa University and different pollen morphology guides were used for the recognition of the pollen types.

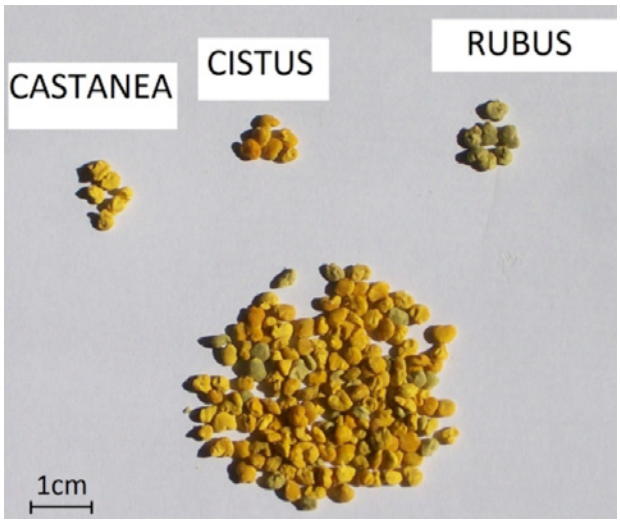

Fig. 1 - Picture of the original blended bee-pollen sample and *Castanea* sp. (yellow), *Rubus* sp. (green) and *Cistus* sp. (ochre) pollen.

2.4 Chemical composition

Dry matter, proteins, lipids and ash quantification were performed for the pollen-load samples according to AOAC guidelines (AOAC, 2000) and values were expressed as percentage on fresh matter basis. Dry matter content was made through gravimetry until constant weight, using oven at 105°C. Protein content was obtained using Kjeldahl method, while lipid content was determined by Soxhlet extractor using diethyl ether as solvent. Ash content was determined by gravimetry until constant weight, using oven at 550°C for one day. Moisture content was obtained by subtracting the dry matter from 100, while total carbohydrates content was determined according to the following formula: Carbohydrates (g) = 100 - [protein (g) + fat (g) + moisture (g) + ash (g)] (Ketkar, Rathore, Lohidasan, Rao, Paradkar, and Mahadik, 2014).

2.5 Bee-pollen extraction and phytochemical characterization

Pollen-loads were separated in three color groups and the mixed pollen sample was made blending the three pollen type in equal part. Bee-pollen extracts were obtained after 1 hour incubation at room temperature in 95% ethanol while being shaken gently. Then samples were centrifuged 10 minutes at 3500 rpm at 4°C and the supernatants were collected and kept in the dark at 4°C.

Total polyphenols were determined by the Folin-Ciocalteu colorimetric method (SINGLETON *et al.*,1999). Briefly, 100 µL of each natural extract were mixed with 500 µL of 0.2 N Folin-Ciocalteu reagent and incubated in the dark for 5 minutes. Then, 400 µL of 0.7 M sodium carbonate (Na2CO3) were added. The absorbance was measured at 760 nm, after 2 hours incubation at room temperature in the dark. Five serial dilutions of gallic acid (0.009, 0,017, 0,043, 0,085, 0.17 mg/mL) were used to obtain the standard calibration curve with the following equation: conc=0.079Abs-0.004 (R^2=0.996). Total polyphenols were expressed as mg of gallic acid equivalents (GAE)/g dry weight (dw).

The aluminum chloride colorimetric method was used for the total flavonoids determination (Kim D.O., Chun, Kim Y.J., Moon, and Lee, 2003). Briefly, 200 µL of extracts were mixed with 800 µL of dH_2O and 60 µL of 5% $NaNO_2$, followed by incubation of 5 minutes at room temperature. Then 60 µL of 10% $AlCl_3$ were added, incubated for 6 min and finally reactions were neutralized with 400 µL of 1M NaOH. Absorb-

ance was measured at 430 nm after 30 minutes of incubation and catechin was used to make the calibration standard curve (0.016, 0.032, 0.063, 0.125, 0.25, 0.5 mg/mL). Flavonoids concentration was obtained from the following calibration curve: conc=0.464Abs-0.005 (R^2=0.998) and expressed as mg catechin equivalent (CE)/g dw.

Flavonols content was measured according to the method described by Romani, Mancini, Tatti and Vincieri (1996). Briefly, 25 μL of extracts were mixed with 225 μL of 10% EtOH, 250 μL of 0.1% HCl in 95% EtOH and 1 mL of 2% HCl. After incubation at room temperature for 30 minutes, the absorbance of the reaction mixture was measured at 360 nm and quercetin was used to make the calibration standard curve (0.0006, 0.00125, 0.0025, 0,0050, 0.0075, 0.01, 0.0125, 0.015 mg/mL). Flavonols content, derived from the calibration curve equation: conc=0.536Abs-0.0001 (R^2=0.997), was expressed as mg quercetin equivalent (QE)/g dw,

Total monomeric anthocyanins were determined according to the pH differential method described by Lee, Durst, and Wrolstad (2005), a spectrophotometric method based on the change in pigmentation pH-dependent of anthocyanins. Absorbance was measured at 520 and 700 nm and anthocyanin concentration was expressed as mg cyanidin-3-glucoside equivalents (C3GE)/g dw (cyd-glu, molar extinction coefficient of 26,900 L cm^{-1} mol^{-1} and molecular weight of 449.2 g mol^{-1}).

2.6 Front-Face Fluorescence spectroscopy analysis

Each pollen sample was studied previous in its bulk state by FF fluorescence spectroscopy, then their ethanolic extracts were studied by UV-Vis absorption and FF fluorescence spectroscopies. The precipitate obtained after the extraction was also studied by FF fluorescence spectroscopy. To recognize the main classes of fluorescence compounds in pollen loads, standard solutions of the reagents reported in paragraph 2.1 were studied by Uv-Vis Absorbance and FF fluorescence spectroscopy.

For bulk analysis pollen loads were finely powdered with a pestle in a mortar. A little amount of powder was put between two quartz windows of 1 mm optical path with the help of few water drops to homogenized the sample (about 1:1 mg/ml sample: water). These quartz windows are held against a support in the spectrofluorometer by a laminar spring. For extraction procedure 2 mL of ethanol were added to 100 mg of pollen loads finely powdered. They were stirred for 30 minutes with a magnetic stir bar and then filtered through filter paper 0.45 mm pore size (Sartorious), to obtain a clear solution. To do it, Spectrum S-25-10 stirred cell device for ultra-filtration was used. A quartz cell with 2 mm optical path was used to record absorbance spectra and low capacity quartz cells with 5 mm optical path was used to record FF fluorescence spectra. The residual material was kindly removed from the filter paper and it was put between two quartz windows of 1 mm optical path. Fluorescence spectra were recorded using a ISA Fluoromax II photon counting spectrofluorometer, with Xenon arc lamp and a device for front-face measurements with a cell holder designed to set the incident angle of the excitation beam at 31°, eliminating or reducing self-absorption effects, light-reflected and scattering (ZANDOMENEGHI *et al.*, 2005). The excitation and the emission slits were 2 and 5 nm, respectively. The integration constant time was 0.5 s and the wavelength increment was 1 nm. The intensity of the spectra was determined as the ratio between the emisson signal (counts per second, cps) and the intensity of light from the excitation monochromator (mA), measured by means of a photomultiplier and a photodiode, respectively. For each sample emission spectra (280 nm < lambda_ex < 550 nm, with a step of 10 nm), excitation spectra (at the wavelength of the maximum position of fluorescence emission spectra, l_{em}) and synchronous spectra (20 nm < Deltalambda < 120 nm, with a step of 10 nm) were recorded. For bulk analysis emission spectra with l_{ex}=650 nm were recorded. Absorbance spectra were recorded with a Jasco V-550 spectrophotomer, between 200 nm and 750 nm with scanning speed of 400 nm/s, band width and data pitch of 1 nm and 0.5 nm, respectively.

2.7 Antioxidant activity

2.7.1 DPPH radical scavenging assay

The free-radical scavenging activity of ethanolic bee-pollen extracts was evaluated using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay (FRASSINETTI *et al.* 2011). The reduction of DPPH radicals was recorded at 517 nm and the radical scavenging activity (RSA) was calculated as percentage of DPPH inhibition according to the following equation: % RSA = $[(A_{DPPH}-A_S)/A_{DPPH}]$ x 100, where A_{DPPH} is the absorbance of DPPH solution and A_S the absorbance of sample. The extract concentration corresponding 50% of DPPH inhibition (EC_{50}) was measured by interpolation from the graph of RSA percentage versus bee-pollen concentration (Morais *et al.*, 2011). Lower EC_{50} values indicate higher antioxidant activities.

2.7.2 Oxygen Radical Absorbance Capacity (ORAC) Assay

The antioxidant capacity of ethanolic bee-pollen extracts was quantified using the oxygen radical absorbance capacity (ORAC) assay, modifying some reagent concentration adapted to our requirements (NINFALI *et al.*, 2005).

The final reaction mixture of our assay contained 0·04 mM fluorescein sodium salt in 0.075

M phosphate buffer, pH 7.4, at diluted sample or 5 mM Trolox. The control was 0.075 M phosphate buffer, pH 7.4. AAPH was used as peroxyl radicals generator and fluorescein as probe. Fluorescein fluorescence decay was read at 485 nm excitation and 514 nm emission using a VictorTM X3 Multilabel Plate Reader (Waltham, MA) and Trolox was used as antioxidant standard. ORAC values were expressed as micromoles of Trolox equivalents (TE)/g dw.

2.8 Statistical analysis

The statistical analysis was performed using GraphPad Prism, version 5.00 for Windows (GraphPad software, San Diego, CA). Assays were carried out in triplicate and results were expressed as mean values ± standard deviation (SD). Differences between bee-pollen samples were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post test. A *p*-value lower than 0.05 is considered as statistically significant. Interdependence between the antioxidant capacity and the phytochemical profile was evaluated by Pearson's correlation coefficient (r).

3. RESULTS

3.1 Palynological analysis

The blended bee-pollen sample resulted to be a mixture of *Castanea* sp. (yellow), *Rubus* sp. (green) and *Cistus* sp. (ochre). Each pollen load owned a homogeneous and monospecific pollen content. The *Castanea* sp. was the most representative (70%), followed by *Rubus* sp. (23%) and *Cistus* sp. (7%).

3.2 Chemical composition

The chemical composition of *Castanea*, *Cistus* and *Rubus* pollen samples is listed in Table 1 and values are expressed as percentage on fresh matter basis. The nutritional content measured is in agreement with literature values (Balkanska and Ignatova, 2012; Carpes, Mourão, Alencar, and Masson, 2009; Nogueira, Iglesias, Feás, and Estevinho, 2012). Significant variations among samples were showed, with the highest protein ($p<0.01$) and lipid ($p<0.001$) content in *Rubus* compared to all other pollens. No difference among groups was found for moisture, dry matter, ash and carbohydrates content (p=ns).

3.3 Phytochemicals profile of ethanolic pollen-load extracts

Pollen-load extracts were screened for total polyphenols, flavonoids, flavonols and monomeric anthocyanins content. Phytochemical profile of *Castanea*, *Cistus* and *Rubus* pollen-load samples is listed in Table 2 and significant differences were found ($p<0.001$). In particular, *Castanea* pollen extracts contained the highest levels of polyphenols (24.75±0.78 mg GAE/g fw), flavonoids (15.86±0.62 mg CE/g fw) and anthocyanins (77.37±2.55 mg C3GE/L), while the highest levels of flavonols (4.93±0.05 mg QE/g fw) were detected in *Cistus* pollen samples. Otherwise, *Rubus* pollen extracts showed the lowest

Table 1 - Moisture, dry matter, proteins, lipids, ash and carbohydrates of pollen-load samples.
[a],[b],[c] Different superscript letters indicate statistical differences among the bee-pollen extracts ($p<0.001$ ANOVA). Assays were carried out in triplicate and results were expressed as mean values ± SD.

	***Castanea* pollen**	***Cistus* pollen**	***Rubus* pollen**	***p***
Moisture (%)	10.75 ± 1.25	12.03 ± 1.01	11.06 ± 1.50	n.s.
Dry matter (%)	89.24 ± 2.22	87.97 ± 2.43	88.31 ± 2.07	n.s.
Proteins (%)	26.57^{a} ± 0.34	25.87^{a} ± 0.29	28.42^{b} ± 0.31	<0.001
Lipids (%)	2.07^{a} ± 0.14	1.92^{ac} ± 0.13	2.83^{b} ± 0.13	<0.001
Ash (%)	2.63 ± 0.18	2.55 ± 0.16	2.85 ± 0.15	n.s.
Carbohydrates (%)	57.98 ± 1.91	57.63 ± 1.59	54.84 ± 2.09	n.s.

Table 2 - Total polyphenols, flavonoids, flavonols and anthocyanins concentration of ethanolic pollen-loads extracts. [a,b,c] Different superscript letters indicate statistical differences among the bee-pollen extracts ($p<0.001$ ANOVA). Assays were carried out in triplicate and results were expressed as mean values ± SD.

	Castanea pollen	*Cistus* pollen	*Rubus* pollen	*p*
Total polyphenols (mg GAE/g fw)	24.75±0.78[a]	21.19±0.24[b]	13.53±0.4[c]	<0.001
Flavonoids (mg CE/g fw)	15.86±0.62[a]	14.21±0.56[b]	5.91±0.27[c]	<0.001
Flavonols (mg QE/g fw)	4.77±0.09[ac]	4.93±0.05[a]	2.52±0.14[b]	<0.001
Anthocyanins (mg C3GE/L)	77.37 ±2.55[a]	57.19±5.84[b]	53.44±2.36[b]	<0.001

content of polyphenols, flavonoids and flavonols. The discrepancies in phytochemical composition observed among the different pollen samples might depend on their botanical origin (ARRUDA *et al.*, 2013; CAMPOS *et al.*, 2008; MORAIS *et al.*, 2011).

3.4 Fluorescence spectroscopy

3.4.1 Bulk analysis

Pollen is a complex matrix and, as a consequence, the fluorescence spectra are characterized by broad and overlapped bands, caused by the presence of many fluorophores, which limit the quantification and identification of all of them. However, it is possible to get several information as well as qualitative features, which can be used as fingerprint of the investigated pollen.

The fluorescence spectra are indeed characterized by three main intervals of excitation wavelength: 280-290 nm, 320-370 nm and 420-480 nm. In Fig. 2a the emission spectra obtained with excitation wavelength (l_{ex}) of 280 nm are reported. Two bands can be recognized: the first one, less intense, is centered at 340 nm for *Rubus* and at 360 nm for *Castanea* and *Cistus* pollens. This band is probably due to the aromatic aminoacids, which can be residues of proteins or free. The second one is the dominant band and its shape is different between *Rubus* and *Castanea*/*Cistus* pollens: it is more intense in the range 420-620 nm for *Rubus* and red shifted of about 40 nm for *Castanea* and *Cistus* pollens. This emission band is probably due to hydroxycinnamic acids, compounds belonging to polyphenols family, fluorescent water soluble vitamins such as B6, B9 and B2, and flavoinoid compounds. All these classes of compounds have two bands of absorption (three in the case of B2 vitamin), the first one centered in the 280-290 nm interval. This hypothesis is supported by the emission spectra obtained at highest l_{ex} 320-370 nm, where the above mentioned substances are excited in their second absorption band; these pollens' bands are indeed less intense but retain the same shape of previous broad band. It is interesting to observe that *Castanea* and *Cistus*'s spectra are again much different from *Rubus* (Fig. 2b), probably due to the presence of characteristic fluorophores in the latter pollen type.

For all pollens, fluorescence intensity decreases until λ_{ex}=400 nm; thereafter, it slightly increases again, to define a new band centered at 530 nm for *Cistus* and *Castanea* and at 510 nm for *Rubus* (λ_{ex}=450 nm). At this wavelength, B2 vitamin has its third absorption band, moreover, other fluorophores are excited, such as xanthophylls and carotenoids derivatives, although they are very weak emitter (JØRGENSEN *et al.*, 1992). Changing the λ_{ex} to 550 nm, only *Cistus* pollen presents a weak fluorescence at about 600 nm, probably caused by the presence of other polar carotenoids derivatives. At higher excitation wavelength, collected to investigate the eventual presence of chlorophyll derivatives (λ_{ex}=650 nm), no relevant fluorescence is recorded.

The synchronous spectra obtained with 60 nm offset (Fig. 2c) underlines the presence of three different bands of absorption and they are useful to compare the pollen types. The fluorescence profile of *Rubus* pollen is much different than that of *Castanea*/*Cistus*' one, especially in the second absorption bands (IIR and IIC respectively). In this spectrum, also the third band differs between *Rubus* and *Castanea*/*Cistus*, centered

at 440 nm (IIIR) and 480 nm (IIIC), respectively. Moreover, the *Cistus* pollen is the only one presenting an absorption centered at 550 nm (IV). All above data are consistent with the emission spectra. Vitamins C and B_3 did not show relevant fluorescence so they resulted undetectable.

3.4.2 Extracts analysis

In order to facilitate the spectral interpretation, an extraction procedure to separate the water soluble fraction from the lipid soluble one was adopted. The solvent used are not strong and the treatment of samples is such to limit the alteration of the matrix, and to use the potentiality of a rapid and direct spectroscopic technique. In the present work ethanolic extracts were studied.

The most interesting region of the UV-Vis absorption spectra is in the visible range (400-500 nm). Here, only *Cistus* extract presents an absorption spectrum with the typical shape of carotenoid or xanthophyll pigments, as shown in Fig. 3a.

Considering fluorescence, the emission spectra at λ_{ex}=280 nm (Fig. 3b) are characterized by a broad band, extended to the interval 340-520 nm. *Castanea* and *Rubus* pollen show maximum emission at about 420-430 nm, while the *Cistus* one shows a different profile with an emission band centered at about 400-410 nm and a shoulder at 520 nm. In this region, the fluorescence is probably due to the hydroxycinnamic acid, that at this wavelength begin to absorb. The polyphenols belonging to the hydroxybenzoic family (e.g. gallic acid, 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, vanillic acid) are not the mainly fluorophores. In fact, they have their absorption and emission maxima included in the interval 280-297 nm and 325-370 nm, respectively.

In Fig. 3b emission spectra of two representative compounds of the hydroxybenzoic's family (gallic and 4-hydroxybenzoic acids) compared with pollen's spectra are reported. The hypothesis of the presence of hydroxycinnamic acid is supported by the emission spectra recorded with highest λ_{ex} (310-340 nm), characterized by the same shape, but higher intensity. In fact, polyphenols belonging to hydroxycinnamic family (e.g. caffeic acid, ferulic acid, p-cumaric acid) have their absorption and emission maxima included in the interval 310-350 nm and 410-440 nm, respectively. Emission spectra of two representative compounds of this group (caffeic and p–coumaric acid) recorded with λ_{ex}=310 nm are reported in Fig. 3c and compared with pollen's spectra. *Castanea* pollen spectrum differs from the two others for the higher intensity.

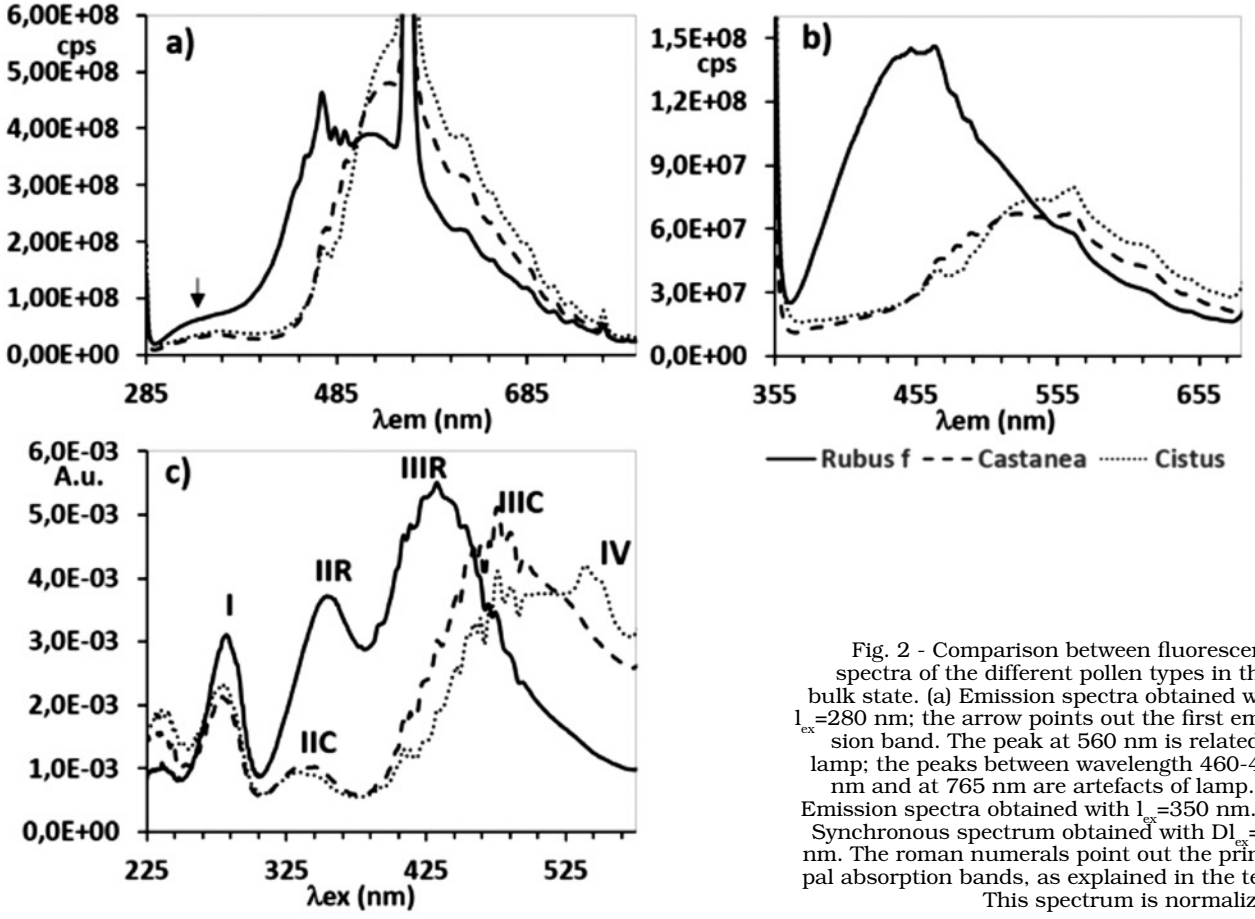

Fig. 2 - Comparison between fluorescence spectra of the different pollen types in their bulk state. (a) Emission spectra obtained with l_{ex}=280 nm; the arrow points out the first emission band. The peak at 560 nm is related to lamp; the peaks between wavelength 460-490 nm and at 765 nm are artefacts of lamp. (b) Emission spectra obtained with l_{ex}=350 nm. (c) Synchronous spectrum obtained with Dl_{ex}=60 nm. The roman numerals point out the principal absorption bands, as explained in the text. This spectrum is normalized.

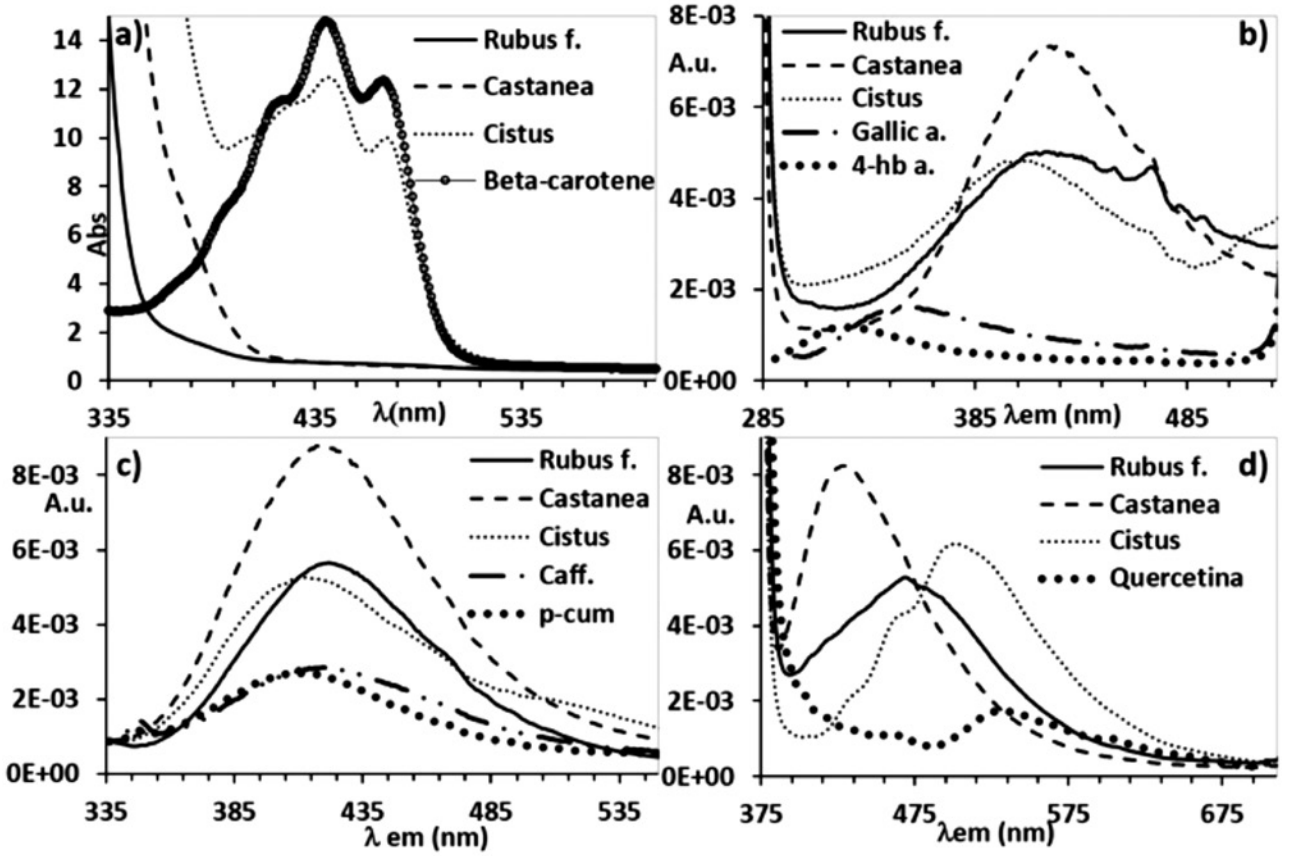

Fig. 3 - Spectra of ethanolic extracts of the different pollen types. (a) Uv-Vis absorpotion spectra (optical path=1 cm) of pollens compare to a b-carotene solution (3.2 mg/ml). Series of emission spectra of pollens compare to: (b) solutions of gallic acid and 4-hydroxybenzoic acid (0.01 mg/ml 90% H_2O-10% CH_3OH) (l_{ex}=280 nm); (c) solutions of caffeic acid and p-coumaric acid (0.01 mg/ml 90% H_2O-10% CH_3OH) (l_{ex}=310 nm); (d) solution of quercetin (20 mg/ml in CH_3OH) (l_{ex}=370 nm). All the fluorescence spectra are normalized. Caffeic and p-cumaric' spectra intensity are multiplied by 2.4.

Considering the excitation spectra recorded with the emission wavelength fixed in the maximum of emission (430 nm for *Castanea*/*Rubus* and 410 nm for *Cistus*) it is clear that their fluorescence is due to different fluorophores. In particular, they are a single fluorophore, with an absorption band centered at 330 nm for *Rubus*, a single fluorophore, with two absorption bands centered at 285 and 330 nm for *Castanea*, and two different fluorophores, centered at 290 and 325 nm excitation wavelength for *Cistus*. This latter hypothesis is confirmed by synchronous spectra obtained with offset 100 nm, in which it is possible to recognize two different contributions with absorption at 295 and 320 nm, respectively.

Changing the λ_{ex} to 350 nm (Fig. 3d), the fluorophores involved are others, thus confirming the complexity of the pollen matrix.

Castanea and *Rubus* samples present the same profile, while *Cistus*' spectrum shows a particular shape characterized by three shoulders, most likely due to three different fluorophores, confirmed by excitation spectra recorded with λ_{em}=460nm. These fluorophores are probably other hydroxycinnamic compounds (in the cases of emission at 430 nm and 460 nm) and flavonoids compounds (in the case of emission at 500-520 nm). For example, the flavonol quercetin has a weak emission, but the shape of its fluorescence spectrum is compatible with that of *Cistus* pollen, as reported in Fig. 3d.

The richness in fluorescent compounds of *Cistus* pollen is confirmed by the synchronous spectrum obtained with offset 120 nm, where it is possible to recognize four bands of absorption, centered at 295-320 nm, 350 nm, 370 nm and 400 nm. In the case of the first broad band it is possible to discriminate the contribution of two fluorophores with offset 100 nm, like previously discussed.

With higher excitation wavelength, collected to check the presence of carotenoids or xanthophyll with front-face fluorescent of liquid extract, no relevant fluorescence was recorded. It is important to underline that there are several xanthophylls and carotenoids derivatives, and only some of them have weak emission, as previous discussed in section "Bulk analysis".

Finally is interesting to analyze the fluorescence spectra obtained for solid samples. For example in Fig. 4 the comparison between emission spectra (λ_{ex}= 280 nm) of *Rubus* in its bulk

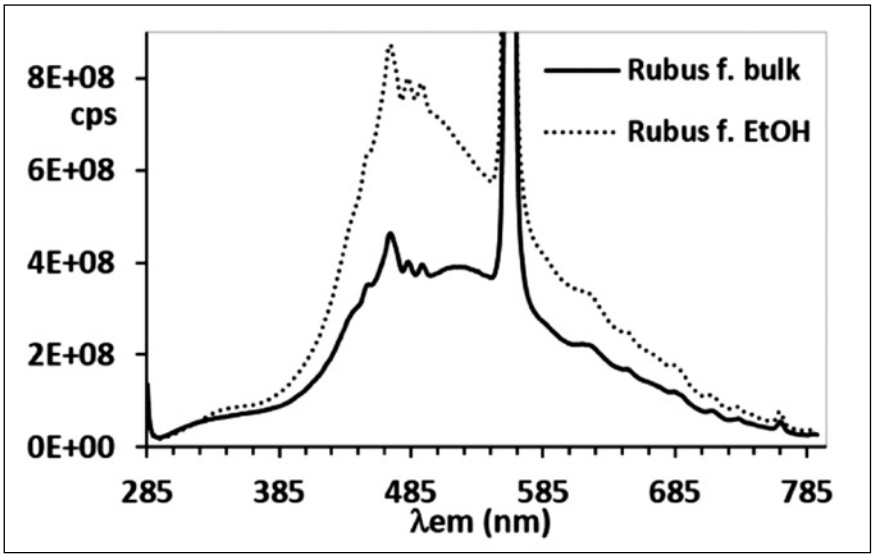

Fig. 4 - Emission spectra obtained with λ_{ex}=280 nm for solid *Rubus* samples differently processed. The dotted line shows the precipitate after ethanol extraction (spectrum intensity is divided by 1.7). The peak at 560 nm is related to lamp; the peaks between wavelength 460-490 nm and at 765 nm are artefacts of lamp.

state with the precipitate after ethanol extraction (dotted line) is reported. The first band due to water soluble aminoacids and proteins, not extracted in ethanol, is present into both spectra. Instead the shape of main broad emission band centered at 500 nm, differs from each other. This can be due to the lack of the classes of compounds extracted with the solvent: hydroxycinnamic acid and flavonol compounds. It is important to remember that fluorescence sensitive changes in relation of the physical state of compounds and their environment (LAKOWICZ, 2006). Although the broad emission band, these results are coherent with extracts' spectra.

3.5 DPPH radical-scavenging activity

The DPPH assay has been widely used to test the free radical scavenging activity of apicultural products, either honey, propolis or bee-pollen. As reported in literature the radical scavenging activity of bee-pollen is very dissimilar among different flower species with a value range of 0-97% that also depends on their chemical composition and solvent extraction (BASUNY *et al.*,2013; LEBLANK *et al.*, 2009; SILVA *et al.*, 2006). We evaluated both percentage of DPPH radical scavenging activity (% RSA) and EC50, the extract concentration providing 50% DPPH inhibition. As shown in Table 3, ethanolic bee-pollen extracts exhibited high free radical scavenging activity with DPPH inhibition values ranged from 37.95±0.19% (EC_{50} = 641.3±11.4 μg/mL) of *Rubus* extracts and 94.45±0.01% (EC_{50} = 215.2±2.7 μg/mL) of *Castanea* extracts.

ANOVA with Tukey post-test showed significant differences among samples with the highest and lowest activity detected in *Castanea* and *Rubus* extracts. As well as radical quenching capacity, *Castanea*, *Cistus* and mixed bee-pollen

Table 3 - ORAC and DPPH assay results of ethanolic pollen-load extracts expressed as μmol TE/g fw and % RSA and EC_{50} (μg/mL), respectively.
[a,b,c] Different superscript letters indicate statistical differences among the bee-pollen extracts (p<0.001 ANOVA). Assays were carried out in triplicate and results were expressed as mean values ± SD.

	***Castanea* pollen**	***Cistus* pollen**	***Rubus* pollen**	**Mixed pollen**	***P***
ORAC (μmol TE/g fw)	544.01±4.75[a]	540.01±17.2[a]	519.45±15.07[a]	677.70±12.92[b]	<0.001
% RSA	94.45±0.01[a]	94.19±0.03[b]	37.95±0.19[c]	94.07±0.03[b]	<0.001
EC_{50} (μg/ml)	215.2±2.7[a]	224±12.3[ac]	641.3±11.4[b]	249.3±6.1[c]	<0.001

possessed similar EC_{50} values, three times lower than *Rubus* extracts, suggesting a lower radical scavenging activity of *Rubus* bee-pollen as antioxidant.

Our results also revealed a strong relation between the antiradical activity and the total phenolics, flavonoids and flavonols content, resulting in a significant positive correlation (r=0.9645, r=0.9888, and r=0.9847, respectively). Lastly, a moderate correlation was obtained between the anthocyanins and the DPPH scavenging activity (r=0.5541).

3.6 Oxygen Radical Absorbance Capacity (ORAC)

The antioxidant capacity of ethanolic bee-pollen extracts was also screened using ORAC assay and expressed as ORAC units (μmol Trolox equivalents/g fw). The ORAC values were listed in Table 3 and ranged from 519.45±15.07 μmol TE/g of *Rubus* fraction and 677.70±12.92 μmol TE/g of mixed bee-pollen. One-way analysis of variance with Tukey's post test showed a significant increase of ORAC values in mixed bee-pollen extracts respect to each one separate fractions ($p<0.001$), suggesting a synergic or additive effect among *Castanea*, *Cistus* and *Rubus* antioxidant compounds. In fact, besides to their specific effects, many antioxidants can interact in synergistic ways, maybe protecting another against oxidative degradation, exhibiting greater antioxidant effects (MĂRGHITAŞ *et al.*, 2009). The results showed no strong correlation between the ORAC values and the anthocyanin (r=0.1524) and flavonoid (r=0.4586) compounds; however, a moderate interdependence was obtained between the antioxidant capacity and the polyphenols (r=0.6638) and flavonols (r=0.5572) content.

4. CONCLUSIONS

In this study, an organic bee-pollen sample from Tuscany was analysed for the first time investigating the botanical origin, the chemical composition, the phytochemicals profile and the antioxidant activity. Different techniques were used. In particular, we propose an original application of FF fluorescence spectroscopy, a promising approach to put in evidence differences and analogies among pollens with different floral origin. This unconventional technique presents the advantage to require no particular sample pre-treatment; moreover, it is economic, fast and easy to use and it could be useful for both further scientific researches and commercial applications. The FF fluorescence results are coherent and uphold the spectrophotometric data obtained in this investigation. Specifically, the differences among *Castanea*/*Cistus* and *Rubus* fluorescence profiles, arisen from bulk study, are comparable and in agreement with the significant differences found in lipids and proteins composition. Furthermore, the ethanolic extracts fluorescence analysis, as well spectrophotometric results, displays a higher content of flavonols and polyphenols in *Cistus* and *Castanea*, respectively. Moreover, the FF fluorescence analysis shows the greater presence of hydroxycinnamic acids than hydroxybenzoic acids, in agreement with literature data (FANALI *et al.*, 2013; KETKAR *et al.*, 2014). Lastly, UV-Vis ethanolic extracts' spectra reveal the presence of carotenoid or xanthophyll pigments only in *Cistus*, confirmed also by bulk state's emission and synchronous spectra λ_{ex}=550 nm and offset 60 nm, respectively).

Besides nutritional and phytochemical composition, the antioxidant and free radical scavenging activity of Tuscan bee-pollen and its monofloral groups was measured. The redox properties of phenolic compounds, especially flavonoid components, play a key role in decomposing peroxides and quenching oxygen, as well as in absorbing and neutralizing free radicals (MĂRGHITAŞ *et al.*, 2009). Moreover, specific bioactive compounds or a combination of them can exert a different antioxidant activity, strongly dependent on structure and polyphenols composition, rather that the phytochemical concentration (MĂRGHITAŞ *et al.*, 2009).

In particular, we showed that mixed bee-pollen exhibit a much better antioxidant activity than the separate fractions with an ORAC value significantly greater than other samples; whereas, *Castanea, Cistus* and mixed bee-pollen showed a comparable DPPH radical scavenging activity greater than *Rubus* antiradical capacity. These results are in agreement with literature data that strongly associate the high ability to neutralize reactive oxygen species to the phenolic compounds structure, mainly flavonoids and cinnamic acid derivatives (LEJA *et al.*, 2007), maybe more representative in *Cistus* and *Castanea* pollen. Furthermore, according to MĂRGHITAŞ *et al.* (2009) the antioxidant effects of bioactive compounds change differently depending on the antioxidant method used. Therefore, we suppose a synergic or additive effect among *Castanea, Cistus* and *Rubus* antioxidant compounds or resulting from new antioxidant substances with greater antioxidant activity.

In conclusion, the use of spectroscopic techniques applied to bee-pollen samples is a suitable tool to underline differences and analogies in their micronutrient composition. The data obtained with different or complementary techniques are coherent and in agreement with literature concerning the variability in chemical composition and antioxidant activity of pollens from different floral sources (LEBLANC *et al.*, 2009; PASCOAL *et al.*, 2014).

Further investigations should be performed to identify and quantify the main fluorescence

compounds present, as well as to investigate on the nature of *Cistus'* pigments. Moreover, future analysis is required to separate and identify the specific profile of antioxidant compounds in mixed pollen sample in order to elucidate, strengthen and confirm the hypothesis of the antioxidant compound's synergic or additive effect or the presence of new antioxidant compounds.

Finally, the results obtained show for the first time the high nutritional value and the good antioxidant activity of Tuscan bee-pollen, which make it an excellent food supplement and a good candidate for a potential nutraceutical product that could be useful in the prevention of free radical associated diseases. As suggested by findings in a previous paper (LUCCHESI *et al.*, 2014), cellular studies could be useful to investigate the intracellular pathways involved in the bee-pollen antioxidant response.

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