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Keywords:	bee pollen, polyhydroxylated-cinnamoyl-spermidine, sporopollenin, ATR-FTIR, HPLC-PDA

An analytical investigation of hydroxylated cinnamoyl polyamines as biomarkers of commercial bee pollen botanical origin

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Abstract

In this study, the phytochemical profile of commercial pollen samples was investigated using different analytical approaches. The samples pollen composition was monitored by optical microscopy. The infrared spectrum of the ethanol extractable material form different pollen samples indicated the specific presence of an aromatic portion in samples dominated by pollens from arboreal species of sweet chestnut pollen grains (*e.g.*, *Castanea sativa* Mill. and *Prunus*). In addition, the UPLC-PDA analysis showed the ubiquitarian presence of an array of different derivatives confirmed as hydroxylated cinnamoyl derivatives of spermidine (major) and of spermine (minor). This profile appeared to be associated with the sample botanical origin. Samples dominated by chestnut honey pollen grains showed the highest content in total amount of these derivatives, with a peculiar profile dominated by the presence of N^1, N^5, N^{10}-tricaffeoyl spermidine. The results showed that their average chemical composition is quantitatively and qualitatively correlated to their botanical origin, suggesting the feasibility of this approach as a practical tool to monitor plant population using honeybee pollen as a bioindicator of the impact of natural and anthropic processes at the local and global level.

1. INTRODUCTION

Honeybees (*Apis mellifera*) provide honey and other highly valuable apiculture products such as bee pollen, wax, propolis, and royal jelly.

Bee pollen is gaining increasing interest in current functional nutrition owing to its well-documented health-beneficial effects that have been correlated to its antioxidant, anti-inflammatory, anticarcinogenic, immunostimulant, and antimicrobial activity (Saisavoey *et al*, 2021). Its administration is suggested both in pediatric and adulthood malnutrition, as well as in cases of loss of appetite, and for the relieve of the adverse symptoms associated to chemo- and radio-therapy, with beneficial effects on the patients (Denisow and Denisow-Pietrzyk, 2016). In addition, recent studies have highlighted the positive impact of bee pollen collection to improve and increase the beekeeper revenue (Hoover and Ovinge, 2018).

Bee pollen is marketed in unprocessed form or after its transformation in tablet or granulate formulations, or as an ingredient of juices, snack bars, etc. In most cases, the label claims do not report the pollen compositional spectrum, usually determined by melissopalynological analysis.

This issue has been raised only in few published studies limited to Spain (Nogueira *et al.*, 2012), Portugal (Pascoal *et al.*, 2014), and Brazil (Valadares *et al.*, 2015), and focused principally on the microbial contamination and generic chemical characterization of commercial bee pollen.

Beside its compositional and nutritional value in terms of macronutrients (Nogueira *et al.*, 2012), minerals, and micronutrients (Orzáez Villanueva *et al.*, 2001; Sattler et al., 2015), only fragmentary knowledge is available in literature regarding its content of secondary bioactive metabolites (Tomás-Barberán *et al.*, 1989; Jannesar *et al.*, 2014). These data would allow to establish a more rational basis for the discrimination of the intrinsic value and quality of the honeybee pollen produced in a particular area compared to pollens from other geographical origins. In this context, it has been argued that the compositional profile of pollenkitt, the most common adhesive material present around pollen grains, may help in the cataloguing of pollen grains, but it is not sufficient (Pacini and Hesse, 2005).

Previous studies demonstrated the presence of phenolamides, or more specifically hydroxy-cinnamoyl-polyamine conjugates (HCPA) (in which the core structure is constituted by spermidine, or by spermine in some species) in the corresponding reproducing organs (anthers) of plants and flowers, as reviewed in **Table S1**. Recently, two new HCPAs, named mongoline and mongolidine, have been isolated and identified from bee pollen of *Quercus mongolica*, thus evidencing the incomplete knowledge about this class of secondary metabolites (Kim *et al.*, 2018). Moreover, a survey of HCPA in the pollen coat of several spermatophytes, mainly dicotyledon species, has been recently reported, suggesting that the metabolic plasticity of plants belonging to these species may rely on trihydroxycinnamoyl spermidine biosynthesis pathway (Elejalde-Palmett *et al.*, 2015). Recent studies have also demonstrated the capacity of some marine species (*Suberea ianthelliformis* and *Pseudoceratina* sp.) to produce phenolamide derivatives with unusual structure (Cazzaniga *et al.*, 2021).

However, a systematic qualitative and quantitative phytochemical profiling of these active substances in commercial bee pollen is lacking. This is noteworthy considering that the profiling of pollen HCPA could potentially represent a discriminative method to easily recognize and identify the botanical species of origin, and to avoid product counterfeiting.

Hence, the aim of this study was to investigate the phytochemical composition of commercial bee pollen of different geographical origins, employing a combination of analytical techniques. The screening was conducted by infrared spectroscopy (IR), high-performance liquid chromatography coupled to diode array UV detection (UPLC-UV/DAD), and high-resolution mass spectrometry (HRMS).

2. Experimental

2.1. Reagents

HPLC and analytical grade solvents and chemicals were purchased from Sigma (Sigma–Aldrich, St. Louis, MO, USA). Formic acid 98–100% was purchased from Fluka (Sigma–Aldrich, St. Louis, MO, USA). Ultrapure water was obtained using a Milli-Q system (Millipore, Merck KGaA, Darmstadt, Germany).

2.2. Samples

Commercial pollen samples were purchased in local shops and supermarkets in Italy, Turkey, Kosovo, Bosnia-Herzegovina, and Romania.

Pollens produced by Aimar Azienda Agricola were collected in Val Maira valley in North-Western Italy, an area known for the abundant presence of chestnut trees (Villar San Costanzo, Cuneo, Italy). The same producer kindly provided pollen from mountain flowers (*Helianthemum* 35% (Cistaceae); Rosaceae >11%; Scrophulariaceae >10%) and from plum tree and spring flowers (*Prunus* 56%, *Quercus robur* 14%, *Salix* 6%, *Acer* 3%).

Multiflora pollens, commercialized by Apicoltura Piana (Castel San Pietro Terme, Bologna, Italy), Luna di Miele (Settimo Milanese, Milan, Italy), and Erbamea (Selci Lama di San Giustino, Perugia, Italy) and HP Italia (Padua, Italy) in bulk size for local shops retail distribution, were imported from Southern Spain (region of Valencia). Romanian pollen was purchased from Parapharm (Cluj-Napoca, Romania). All other samples were purchased in local shops or street markets.

2.3. Sample preparation

Pollen samples (3.0 g) were extracted with ethanol (100 mL). After sonication (30 min), an aliquot (1 mL) of the homogenous suspension was collected for optical microscopy evaluation (see next paragraph), the resting insoluble material was separated by centrifugation (15000 rpm, r = 10 cm; 25155 G-Force), and the solvent layer was filtered on paper (0.2 µm, Whatman®). A 1.0 mL aliquot of this solution was directly submitted to UPLC®-PDA and HPLC-MS analyses, while the remaining

solution was evaporated under reduced pressure, and the dry residue weighted for extraction yield calculation and infrared (IR) spectrum acquisition.

2.4. Optical microscopy

The pollen suspension (previous paragraph) was dried over a heating plate at 40 °C (10 min), the corresponding dry residue was included in Kaiser's glycerol gelatin and the sample protected with a cover glass. Pollens were visualized through optical microscopy (400× and 1000×) using a SWIFT SS300B-25-EP1 microscope equipped with a 1.3 ocular camera assisted by the Easyview software ver. 1.20.08.041615 (Swift Optical Instruments, Schertz, Texas, USA). Pollen type was identified using an in-house pollen reference collection as well by comparison with on-line available databases and literature references (https://www.paldat.org/) (von der Ohe and von der Ohe, 2000). The corresponding pollen nomenclature was adopted according to Persano Oddo and Ricciardelli d'Arbore (1989).

2.5. UPLC®-PDA analysis

Analyses were performed using an ACQUITY UPLC® system (Waters Corporation, MA, USA) equipped with a quaternary pump, an autosampler, a column thermostat compartment, and a photodiode array (PDA) detector. Chromatogram visualization and data extraction were done using the Empower 3 software. Separations were carried out using a BEH C18 column (2.1mm x 50 mm, 130 Å) and a binary gradient using aqueous formic acid (0.1%, v/v) (A) and formic acid (0.1%, v/v) in acetonitrile (ACN) (B) at the total flow rate of 0.3 mL/min. Gradient program: 0-2 min, B = 10%; 2-15 min, B from 10% to 30%; 15-20 min, B = 30%; re-equilibration time: 2 min. Injection volume: 1.0 µL. Column temperature: 30 °C. Observation wavelength: 300 nm. Results were expressed as equivalent *p*-hydroxycinnamoyl propylamide (pHCAPA) synthesized as described in supplementary material. The semi-quantitative total content of HCPA was determined by integration of the relevant peaks in the retention time (RT) interval RT=10-17 min (observation λ=300 nm), and by subsequent

comparison of this area with those of a calibration curve constructed by injection of different amounts of standard pHCAPA (concentration range: 1-100 ng pHCAPA; LOD=0.67 pg injected pHCAPA; LOD=2.01 pg injected pHCAPA; coefficient of variation intraday CV% < 2%, interday CV% < 7%).

2.6. HPLC-HRMS analysis

Samples were analyzed at Unitech COSPECT (University of Milano, Italy) using a Q-TOF Synapt G2-Si (Waters Corporation, MA, United States) instrument equipped with a binary pump, an autosampler, and a UV-DAD detector operating in the 200-400 nm range. Column for analytical separations: Kinetex™ C18 column, particle size 2.6 µm, pore size 100 Å, 100 × 4.6 mm. Solvent system: A = 0.1% formic acid in H_2O and B = 0.1% formic acid in ACN, flow rate =1.6 mL/min. Gradient: 0-3 min, B = 5%; 3-15 min, from B = 5% to 60%; 15-20 min, B = 60%. Injection volume: 10 µL. Source conditions: negative ion mode, capillary voltage 2 kV, sampling cone 15-80, source heater temperature 100-120 °C, desolvation temperature 120-300 °C, desolvation gas (N_2) flow rate 500 L/hr. Leucine enkephalin (Waters) was used as a lock-mass compound, and data were processed with MassLynxTM V4.2 software (Waters).

Compound identification was done based on spectroscopic and accurate mass spectrometric measurements, comparison of chromatographic RT values and by comparison with available literature data.

2.7. Attenuated total reflectance Fourier-transform infrared (ATR-FTIR) spectroscopy

ATR-FT-IR spectra of pollen ethanol extracts were recorded in the 400-4000 cm^{-1} range, using an Alpha spectrometer equipped with an ALPHA's Platinum single reflection diamond ATR unit (Bruker Optics, Milan, Italy). Number of scans n = 25.

3. Results

3.1. Bee pollen characteristics

The main geographical and organoleptic characteristics of the commercial pollen samples are summarized in **Table S2**.

Their visual appearance was characterized by different degrees of color differences, reflecting the biodiversity of pollen-producing plants in the corresponding harvesting areas.

Yellow pollen loads (from dark yellowish to bright yellow) were the most represented in all samples, followed by light green, reddish, blue, violet, purple and grey pollen loads.

Representative microscopic images for morphological analysis of the pollen populations are reported in supplementary file **Fig. S1-S17**.

3.2. Bee pollen ethanol extract yields

The pollen ethanol extraction yielded characteristic, yellow-to-reddish, semi-solid and sticky dry residues, amounting from a maximum of 11.86% (w/w) in sample P07 to a minimum of 3.78% (w/w) in P09 (dominant pollen type *Cistus ladanifer*), with a mean percentage content of 8.00 ± 2.28% (w/w) (**Fig. S18**). No evident association was observed between the extraction yield and the geographical or botanical origin.

3.3. Pollen ethanol extracts: ATR-FTIR and chemometric analysis

The chemical fingerprint of the ethanol extracts was evaluated by ATR-FTIR to describe the major molecular features of this material.

The raw spectra of each pollen sample are reported in supplementary material **Fig. S19**.

The profiles indicated a composition dominated by sporopollenin-like substances: in samples P02 and P05-P08, vibrational absorption profiles with different inter-band relative absorbance ratio at around 1720-1730 cm^{-1} (lipids C=O stretch), 1650 cm^{-1} (amide I: C=O stretch), 1605 cm^{-1} (aromatics), 1580 cm^{-1}, 1510 cm^{-1} (aromatics), and 1430-1440 cm^{-1}, were easily detectable (**Fig. 1A**).

Interestingly, these vibrational bands were only barely detectable or undetectable in all other samples (P01, P03, P10-P17, **Fig. 1B**).

PCA was performed as a multivariate display method on the FT-IR spectra, after column centering, in order to extract the useful information embodied within the data and to visualize the data structure. **Fig. 2** shows the PCA score plot in the space of the first two PCs explaining the 85% of the total variance, and **Fig. 3A** is showing the score plot on the space PC1 versus PC3.

Looking at these score plots, it can be noted that pollen samples partially differ according to their geographical origin: the clusters are not clearly separated but samples from the same country form areas of higher density. In **Fig. 3A** it is possible to recognize a cluster for the Kosovo samples, all of which at negative value on PC1 and positive of PC3 (except sample P13).

Fig. 3B shows the score plot on the space PC1 versus PC3, but in this case samples are coloured according to their total HCPA content. By investigating this plot, it is clear that samples P02, P04, P06 and P07 have a higher value of HCPA, and it is interesting to notice that all these samples came from Italy, and they contain *C. sativa* as main pollen type.

Considering that samples with very positive score along the PC1 are all Italian and belong to the same botanical species (*C. sativa*), the profile of the loadings on PC1 has been evaluated (see **Fig. 4**) to investigate which spectral regions are most significant in characterizing the samples on the basis of their botanical origin; the frequency range 1900-1200 cm^{-1} of commercial bee pollen samples shows the higher loadings on PC1 confirming this spectral band as very informative for the botanical characterization of the pollen samples.

3.4. Pollen ethanol extracts: UPLC®-PDA and HPLC-HRMS analyses

The phytochemical fingerprint of the commercial bee pollen ethanol extracts was evaluated by UPLC®-PDA (**Fig. S20)**.

At visual examination, the results allowed to recognize at least four representative chromatographic patterns appearing consistently throughout the analyzed samples (**Fig. 5A-D)**: samples of Spanish

origin (P01, P09; *Quercus robur* main pollen type) had chromatographic profiles of type A; samples with composition dominated by chestnut pollen had type B profiles, showing a specific peak at around RT = 10.8 min; most samples displayed a type C chromatographic profile characterized by the presence of a dominant peak at around RT = 14.2 min; in some samples (P10, P12, P15, P17; main pollen types from crops) the type C profile was implemented by the significant presence of a set of additional peaks in the RT range 15.5-16-5 min (**Fig. S20**).

Beside minor peaks in the low retention time (RT) range (5-20 min), with UV spectra typical of glycosylated flavone structures, all chromatograms displayed the presence of a variable number of major peaks between RT = 11.9 min and RT = 13.9 min (**Fig. 5**).

Most peaks were generated by substances absorbing in the UV spectral range 300 nm<λ<320 nm, indicating the presence of *p*-coumaroyl and caffeoyl moieties in their structures (**Table 1**).

Small but significant variations (blue-shift, Δλ~-15 nm) from the typical absorption maxima of the respective free and ester bound cinnamic (λ_{max} = 280 nm), coumaric acid (λ_{max} = 300 nm) and caffeic acid (λ_{max} = 325 nm) indicated their probable linkage to aliphatic portions through amide groups, rather than ester groups.

The proposed corresponding structural identifications were supported by the spectroscopic and accurate mass spectrometric data reported in **Table 1** (Yang *et al.*, 2019). Peak 1**,** identified as N^1,N^5,N^{10}-tricaffeoyl spermidine (tri-C-Spdm) **(1)**, was characteristic of samples P02, P04, and P06 with pollen spectra dominated by *C. sativa* pollen (>90%).

Peaks 2, 3, and 4 generated by the homologs N^1,N^{10}-di-caffeoyl, N^5-*p*-coumaroyl spermidine (di-C-Cu-Spdn) **(2)**, N^1,N^{10}-di-*p*-coumaroyl, N^5-caffeoyl spermidine (C-di-Cu-Spdn) **(3)**, and N^1,N^5,N^{10}-tri-*p*-coumaroyl-spermidine (tri-Cu-Spdn) **(4)** respectively, with an approximate 1:2:1 quantitative ratio, were found to characterize samples P01 and P09. The composition of these samples, all originating from Southern Spain, reflected the dominant presence of *Quercus* pollen grains, previously reported to produce the above identified species (Bokern *et al.,* 1995).

The UPLC®-PDA profile of most samples (P03, P05, P07, P08, and P07-P14) was dominated by the presence of peak **4**, generated by tri-Cu-Spdn **(4)**.

The set of peaks 9-12 (**Fig. 5D**, sample P12), attributed to isobaric isomers (probable photoisomers) of the HCPA N^1, N^5, N^{10}, N^{14}-tetra-*p*-coumaroyl-spermine (**6**) (**Fig. S21**), were indicative of the presence of significant proportions of pollen grains from the Asteraceae family belonging to the tribes Anthemideae and Cichoriaceae (*e.g. Taraxacum*).

The total area of these peaks (10 min<RT<18 min) displayed different magnitudes of intensity, as reflected by their semi-quantitative analyses, in which the peak-generating substances were quantified equivalent pHCAPA (**Fig. S21**).

HCPA were present in pollen samples at concentrations ranging from 15.73 ± 3.79 mg_{pHCAPA}/g in sample P06 (sweet chestnut dominant) to 0.79 ± 0.01 mg_{pHCAPA}/g in sample P09 (*Cistus* dominant), with an overall mean content of 5.56 ± 3.79 mg_{pHCAPA}/g (**Fig. S22**).

The total HCPA concentration (**Fig. S22**) showed no significant correlation with the yield (% w/w) of ethanol-soluble substances (**Fig. S18**). Sample P09, in which *Cistus ladanifer* (Cistaceae), *Erica* (Ericaceae) and *Genista* (Fabaceae) were identified as the main pollen, followed by, showed the presence of detectable amounts of hydroxycinnamic/polyamines derivatives.

In the present study we observed variable proportions of hydrophobic substances structurally related to sporopollenin, the main constituent of the outer coating of a pollen spore.

Due to its sticky and adhesive properties, this mix of substances is supposed to be highly important for the pollination and pollen collection processes, providing the extra adhesive force that allows the pollen grains to remain attached to the honeybee hair, helping to increase bee pollen production (Amador *et al.*, 2017). Results obtained by chemometric elaboration of data from the ATR-FTIR analysis of this material provided evidence that specific components may be responsible for the quantitative and qualitative macroscopic differences observed among the samples.

In good accordance with these observations, the results of this study highlighted the diffused presence of species related to HCPA derivatives (with spermidine as the major representative of their amine portion) in the bee pollen content, with a marked quantitative variability.

Most notably, the results suggested the potential utility of HPLC-UV/PDA for the quick and easy classification of the sample botanical origin, which, in turn, appears to be strictly correlated to its potential nutritional value. Through the application of this analytical technique, one could easily determine the presence of pollen types furnishing high concentrations of HCPA, with a structure based on spermidine (*e.g.*, sweet chestnut, oak and plum tree) and spermine (*e.g.*, Asteraceae species such as sunflower and dandelion).

HCPAs belong to the family of phenolamide alkaloids, a class of polyamines *N*-substituted with hydroxy-cinnamoyl groups (cinnamoyl, *p*-coumaroyl, caffeoyl and feruloyl substituents) at both the primary and secondary amine groups of spermidine.

Their biosynthesis is regulated by the activity of species-specific acyltransferase enzymes, such as Spermidine disinapoyl transferase (SDT), Spermidine dicoumaroyltransferase (SCT), and Spermidine acyltransferase (DH29), which selectively catalyze the acylation at the terminal amino groups, thus generating the corresponding N^1-N^{10} di-substituted spermidine derivatives.

An additional action of Spermidine hydroxycinnamoyltransferase (SHT) consists in the acylation at the spermidine secondary N^2 atom, ultimately leading to the formation of tri-substituted spermidine trihydroxy-cinnamoyl derivatives (Elejalde-Palmett et al., 2015). Of note, sample P09, characterized by a pollen spectrum dominated by pollens produced by *Cistaceae* species, was found to have the lowest content in HCPA; this in good agreement with the previously reported SHT enzyme disappearance in the plant order Malvales (Elejalde-Palmett *et al.*, 2015).

N^1, N^2, N^3-tri-coumaroyl-spermidine was identified so far as the typical hydroxyl-cinnamic derivative in *Rosaceae* (Strack et al., 1990), while earlier studies reported the presence of di-hydroxycinnamoyl-spermidines in pollen from *Alnus glutinosa*, *Betula verrucosa*, *Pterocarya fraxinifolia*, *Corylus avellana*, and from species of the *Fagales* family (Meurer *et al.*, 1986, 1988a, 1988b). The exact

hydroxycinnamoyl:spermidine stoichiometric ratio in the conjugates has not been determined for other plant species (Martin-Tanguy *et al.*, 1978).

In the present study, HCPA conjugates based on spermine as the amine portion were identified in pollen loads attributed to the tribes Anthemideae and Cichoriaceae, the latter including the endemic species chicory and dandelion.

These results agreed with those reported by Delporte et al. (2018), who demonstrated the specific replacement of spermidine HCPA with N^1,N^5,N^{10},N^{14}-tetra-*p*-coumaroyl-spermine in the pollen coat of plants belonging to the *Asteraceae* family, including *Cichorium* and *Taraxacum*. This substitution is supposed to be due to the action of the recently identified enzymes CiSHT1 and CiSHT2 (Delporte *et al.*, 2018).

A recent study provided *in vitro* evidence that the bee pollen components most affected by gastrointestinal digestion are HCPAs (Aylanc *et al.*, 2021), supporting the hypothesis that, upon intake, these derivates may be readily converted to absorbable free polyamines (mainly spermidine) and phenolic acid counterparts.

In this context, translational evidence suggested the role of spermidine in aging in humans (Kiechl *et al.*, 2018) and in neuroprotection against age-related neurodegenerative phenomena in insects (Krame *et al.*, 2013; Gupta *et al.*, 2013, 2016), and additional recent findings associated multiple mechanistic pathways to the protective effect of spermidine administration in a rat model of metabolic dysfunction during overnutrition (Liao *et al.*, 2021).

Hence, the important role of bee pollen as a dietary source of HCPA derivatives is highlighted by their potential ability to act as carriers of doses of polyamides in social insects (honeybees), a concept that may well be translated to humans.

4. Conclusion

In this study, the composition of commercial pollen from different geographical and botanical origin has been investigated using a combination of ATR-FTIR and UPLC-PDA/HPLC-MS techniques. The

results showed that their average chemical composition is quantitatively and qualitatively correlated to their botanical origin, suggesting the feasibility of this approach as a practical tool to monitor plant population using honeybee pollen as a bioindicator of the impact of natural and anthropic processes at the local and global level.

Samples with pollen spectra dominated by chestnut pollen were those endowed with the highest content of ethanol-extractable sporopollenin material and HCPA derivatives, the latter with a peculiar phytochemical profile dominated by the phenolamide tri-C-Spdm.

These results also emphasize the importance of HCPA-rich pollen types, such as chestnut pollen, as a dietary source of the potentially beneficial biogenic amines spermidine and spermine.

Hence, further future studies based on a higher number of samples and focused on the *in vivo* HCPA metabolism, absorption, disposition, and bioactivity are warranted.

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Declaration of interest

Declarations of interest: none.

Data availability

The data that support the findings of this study are available on request from the corresponding author.

Ethical guidelines

Ethics approval was not required for this research.

Citations

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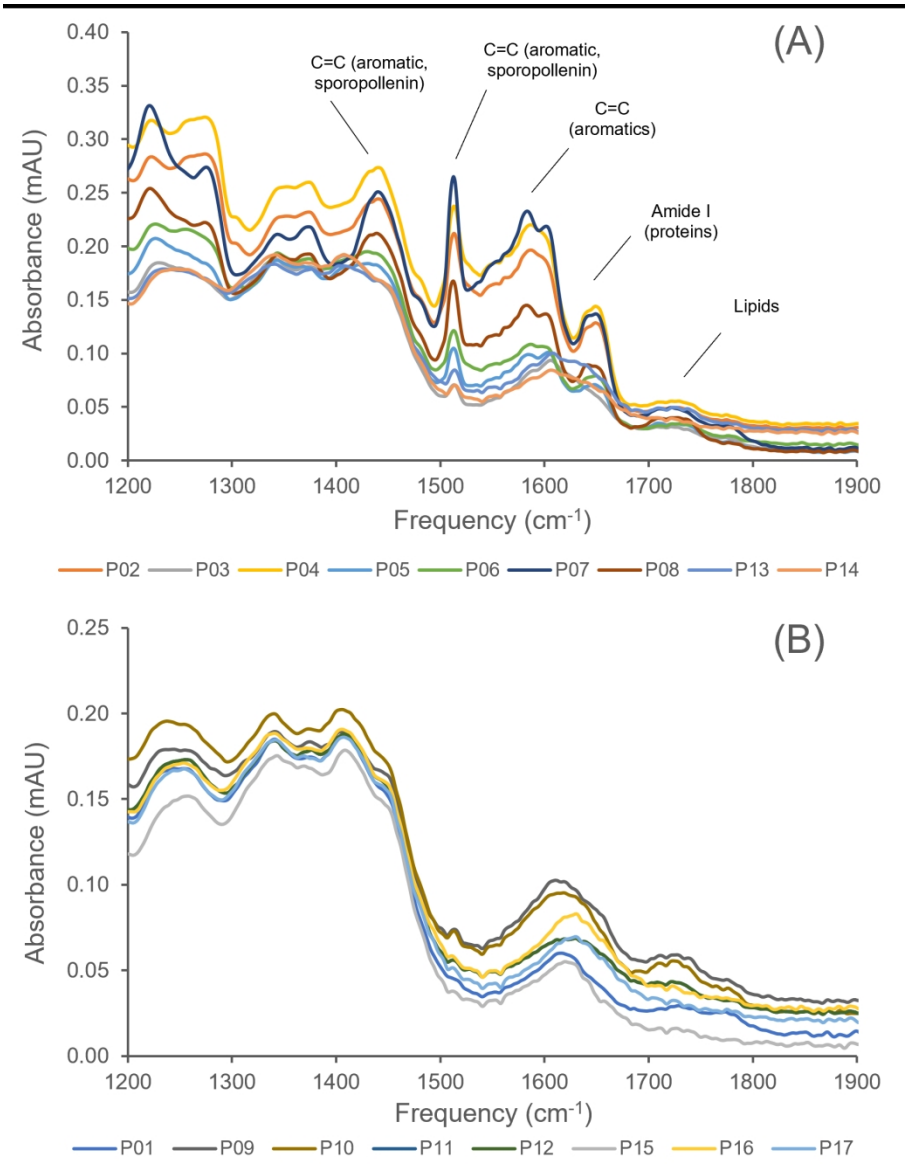

Fig. 1. ATR-FTIR expanded spectral region at frequency range 1900-1200 cm^{-1} of commercial bee pollen samples ethanol extract of different geographical and botanical origin: P02, P05-P08 upper panel (A); P01, P03, P10-P18 lower panel (B).

146x194mm (330 x 330 DPI)

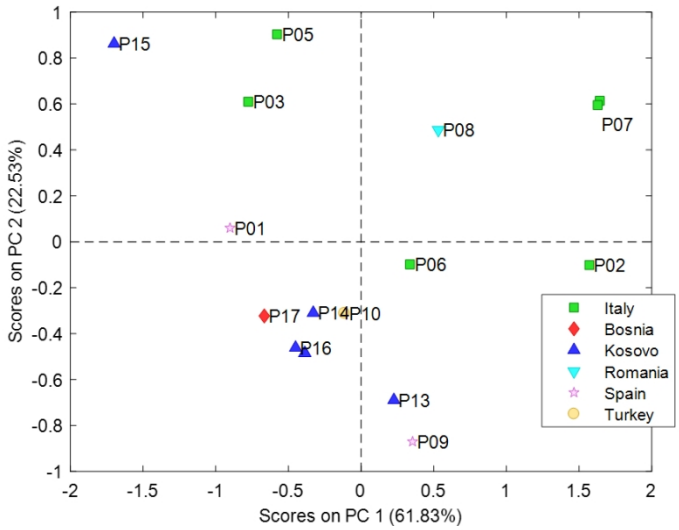

Fig. 2. Score plot -PC1 Vs PC2.

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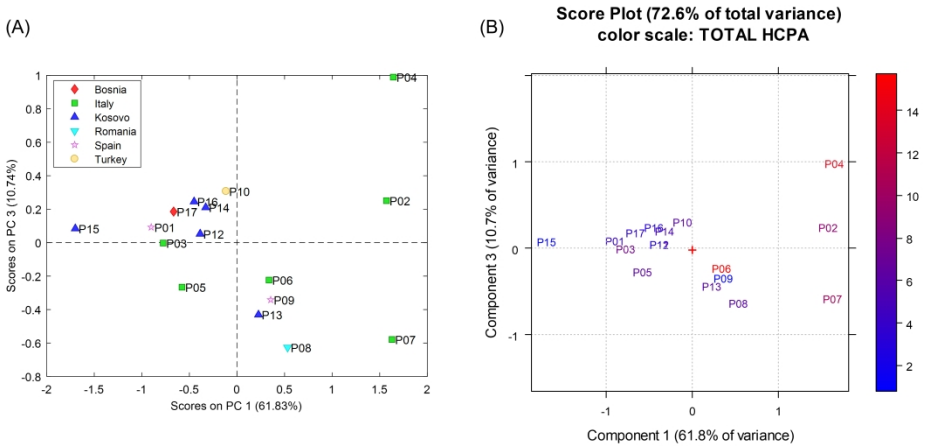

Fig. 3. Score plot -PC1 Vs PC2.

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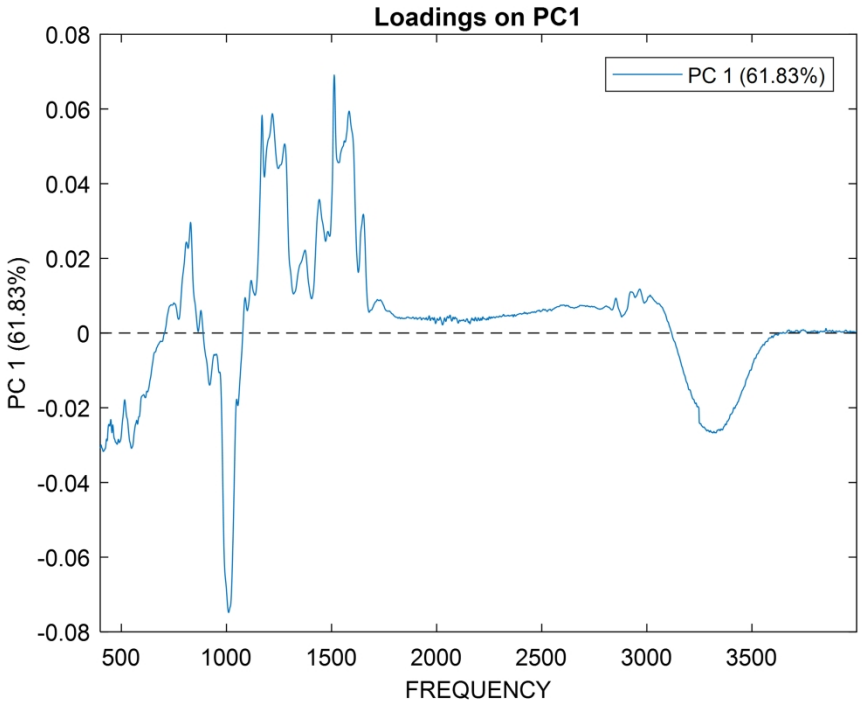

Fig. 4. Loading profile on PC1.

260x190mm (330 x 330 DPI)

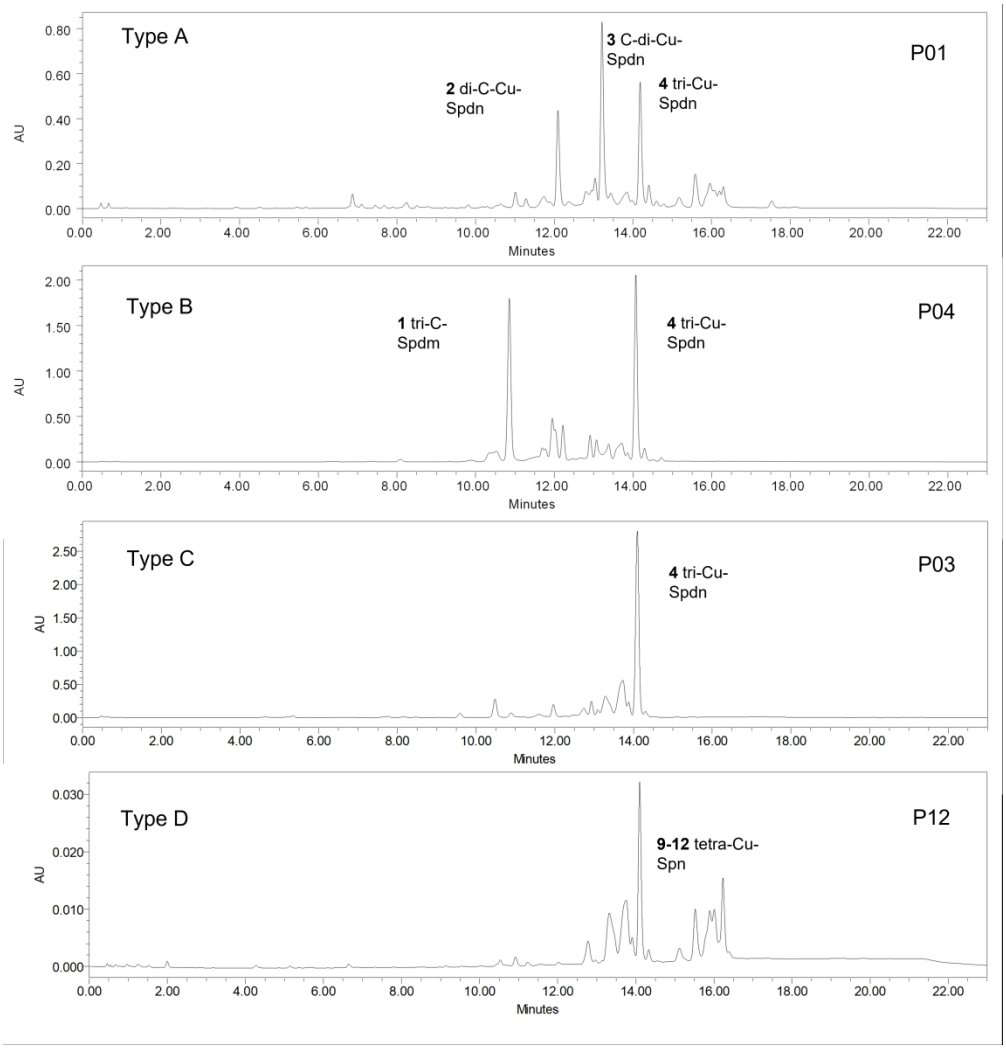

Fig. 5. UPLC®-PDA profiles of selected commercial pollen samples (λ=300 nm).

300x312mm (330 x 330 DPI)

Peak	RT (min)	λ_{max}	$(M-H)^-$ m/z_{exp}	$(M-H)^-$ m/z_{calc}	Molecular formula	Structure assignment
1	29.7	319, 290 sh	630.2431	630.6649	$C_{34}H_{37}N_3O_9$	tricaffeoyl-spermidine
2	33.1	300	614.2507	614.6655	$C_{34}H_{37}N_3O_8$	dicaffeoyl-*p*-coumaroyl-spermidine
3	36.9	298	598.2554	598.6661	$C_{34}H_{37}N_3O_7$	caffeoyl-di-*p*-coumaroyl-spermidine
4	41.1	306	582.2548	582.6667	$C_{34}H_{37}N_3O_6$	tri-*p*-coumaroyl-spermidine
5	41.9	300	612.2695		CHON	Spermidine-derivative (Under definition)
6	42.5	310	642.2809		CHON	Spermidine-derivative (Under definition)
7	43.7	318	672.2897		CHON	Spermidine-derivative (Under definition)
8	41.2	301	785.3558	785.9038	$C_{46}H_{50}N_4O_8$	N^1,N^5,N^{10},N^{14}-tetra-(*p*-coumaroyl)-spermine
9	45.8	275	785.3558	785.9038	$C_{46}H_{50}N_4O_8$	N^1,N^5,N^{10},N^{14}-tetra-(*p*-coumaroyl)-spermine
10	47.5	289	785.3558	785.9038	$C_{46}H_{50}N_4O_8$	N^1,N^5,N^{10},N^{14}-tetra-(*p*-coumaroyl)-spermine
11	48.2	294	785.3558	785.9038	$C_{46}H_{50}N_4O_8$	N^1,N^5,N^{10},N^{14}-tetra-(*p*-coumaroyl)-spermine
12	49.8	301	785.3558	785.9038	$C_{46}H_{50}N_4O_8$	N^1,N^5,N^{10},N^{14}-tetra-(*p*-coumaroyl)-spermine

SUPPLEMENTARY MATERIAL

An analytical investigation of hydroxylated cinnamoyl polyamines as biomarkers of commercial pollen botanical origin and quality

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Keyword: bee pollen, polyhydroxylated-cinnamoyl-spermidine, sporopollenin, analysis.

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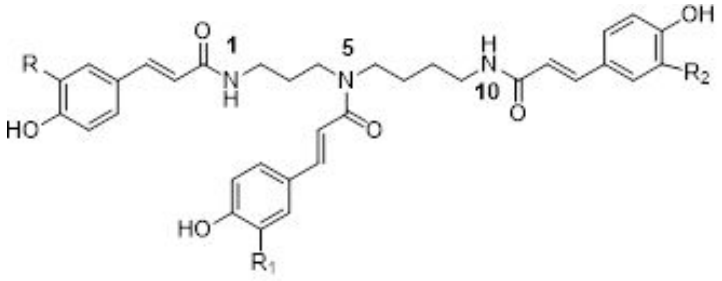
Tab. S1. Botanical origin of the most known pollen spermidines



R	R1	R2	Compound	Pollen botanical origin
-H	-H	-H	N^1, N^5, N^{10}-tri-*p* coumaroyl spermidine	Sunflower (Lin and Mullin, 1999) Apple tree (Elejalde-Palmett et al., 2015) Daimyo oak (Bokern et al., 1995) Green tea (Yang et al., 2012) Thale cress (Handrick et al., 2010)
-H	-OH	-OH	N^1-*p*-coumaroyl-N^5, N^{10}- di-caffeoyl spermidine and related photoisomers	Daimyo oak (Bokern et al., 1995)
-H	-OH	-H	N^1, N^{10}-di-*p*-coumaroyl-N^5, caffeoyl spermidine and related photoisomers	Daimyo oak (Bokern et al., 1995)
-OH	-OH	-OH	N^1, N^5, N^{10}-tri-caffeoyl spermidine	Daimyo oak (Bokern et al., 1995) Apple tree (Elejalde-Palmett et al., 2015)
$-OCH_3$	$-OCH_3$	$-OCH_3$	N^1, N^5, N^{10}-tri-feruloyl spermidine	Green tea (Yang et al., 2012) amaryllis (Youhnovski et al., 2001)
$-OCH_3$	-H	$-OCH_3$	N^1, N^{10}-di-feruloyl-N^5- *p*-coumaroyl spermidine and related photoisomers	Green tea (Yang et al., 2012) Thale cress (Handrick et al., 2010)
-H	-H	$-OCH_3$	N^1, N^5-di-*p*-coumaroyl-N^{10}- feruloyl spermidine and related photoisomers	Green tea (Yang et al., 2012) Thale cress (Handrick et al., 2010)

Tab. S2. Geographical and organoleptic characteristics of the analyzed commercial pollen samples

I.D.	Producer-vendor	Origin	Main pollen types	Label	Color
P01	Apicoltura Piana	Spain	*Quercus* (+++) Campanulaceae Compositae A, T *Echium*	n.s.	Multicolor
P02	Aimar	Italy	*C. sativa* (+++) *Rubus* (+)	Chestnut	Yellow
P03	Aimar	Italy	*Prunus* (+++) *Quercus rubur* (++) *Malus/Pyrus* (++) *Salix* (+) *Acer* (+)	Prunus and spring flowers	Yellow brownish
P04	Beekeper		*C. sativa* (+++) *Rubus* (+)	n.s.	Yellow
P05	Aimar	Italy	*Helianthemum* Rosaceae Scrophulariaceae	Mountain flowers	Multicolor
P06	ADI	Italy	*C. sativa* (+++) *Rubus* (+)	n.s.	Yellow, greenish
P07	ADI	Italy	*Prunus* (+++)	n.s.	Yellowish, Brownish
P08	Parapharm	Romania (west)	*Trifolium* *Taraxacum* (Asteraceae)	n.s.	Multicolor
P09	Erba Mea	Spain (south)	*Cistus ladanifer* *Erica, Genista*	n.s.	Yellow
P10	Mindivan	Turkey	*Aesculus carnea* *Zea Mays*	n.s.	Yellow brownish/brown
P11	Miracle products	Kosovo	*Aesculus carnea*	n.s.	Yellow/greenish
P12	Miracle products	Kosovo	*Heliantuus annuus*	n.s.	Yellow/orange
P13	BIO HERBS	Kosovo	*Trifolium repens* *Trifolium spp* *Heliantuus annuus*	n.s.	Greenish
P14	Beekeeper/street market	Kosovo	*Trifolium repens,* *Trifolium spp* *Filipendula vulgaris*	n.s.	Yellow greenish
P15	Beekeeper/street market	Kosovo	*H. annuus* *Zea mays* *Melampyrum pratense*	n.s.	Yellow brownish
P16	Beekeeper/street market	Kosovo	*Polygonatum odoratum* *Fumana*	n.s.	Yellow brownish
P17	GA-ME-HA	Bosnia-Herzegovina	*Ranunculus acris* *Avena sativa*	n.s.	Yellow brownish/brown

Fig. S1. Sample P01. Oak (*Quercus)* dominant pollen type. Optical microscopy (magnification 100×).

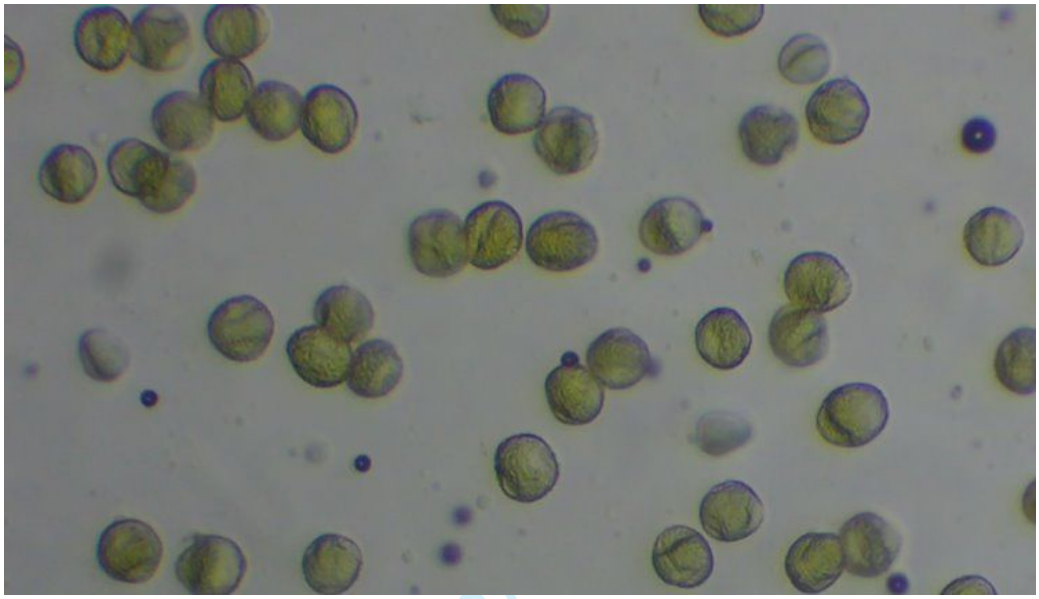


Fig. S2. Sample P02. Chestnut (*Castanea sativa*) dominant pollen type. Optical microscopy (magnification 100×).

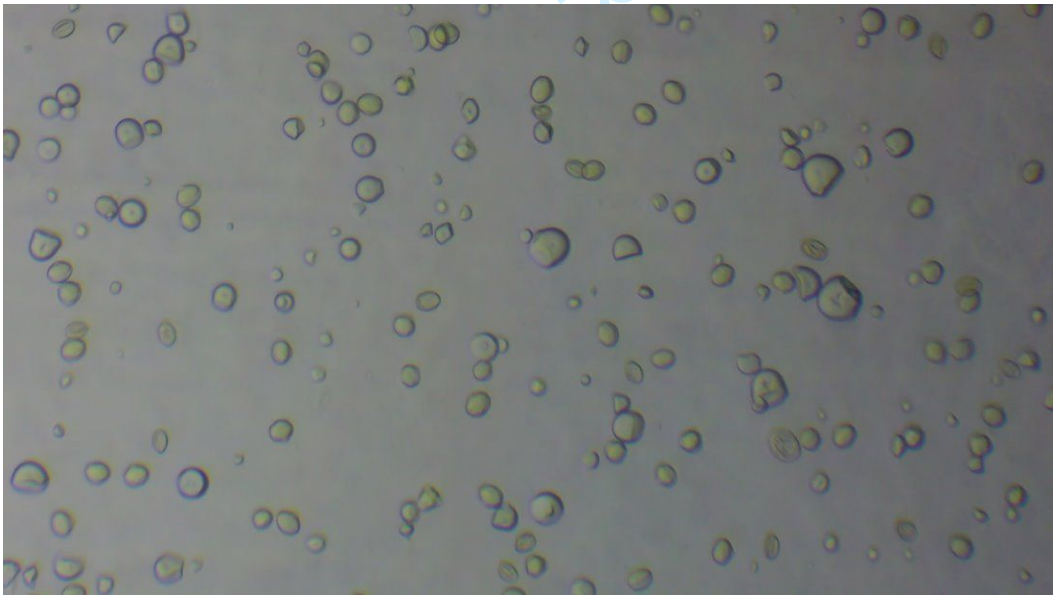


Fig. S3. Sample P03. Plum tree pollen (*Prunus*, main pollen) dominant pollen type. Optical microscopy (magnification 100×).

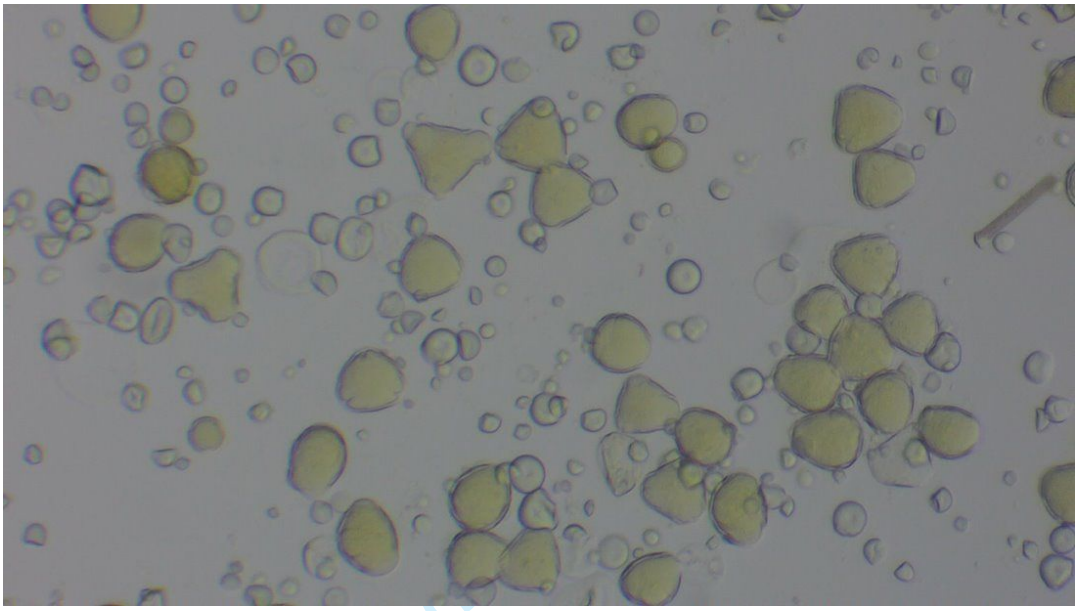


Fig. S4. Sample P04. Chestnut (*Castanea sativa*) dominant pollen type. Optical microscopy (magnification 100×).

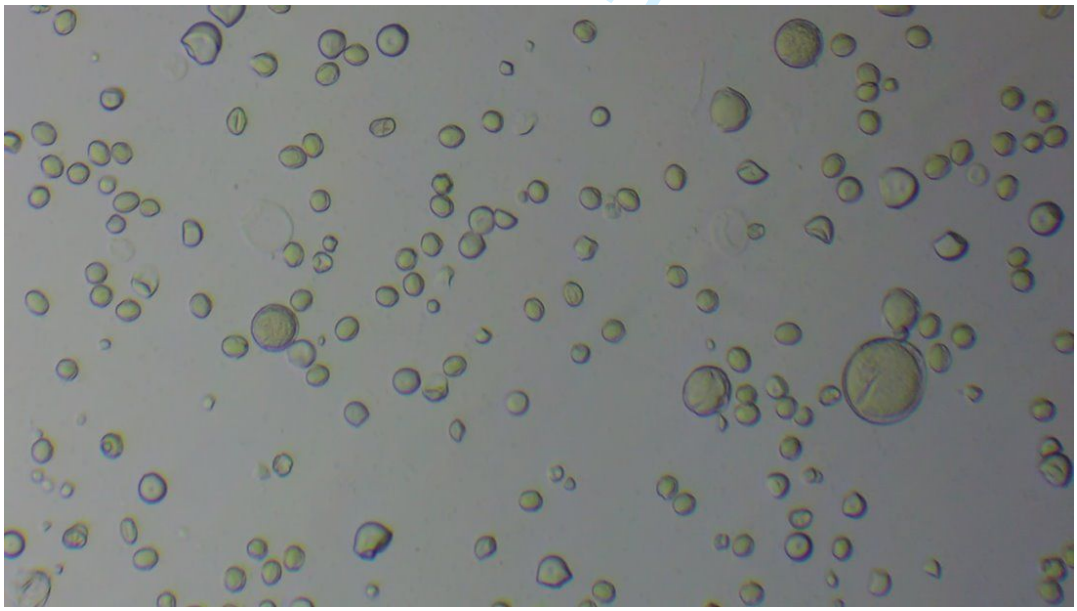


Fig. S5. Sample P05. Mountain flowers. No dominant pollen type.

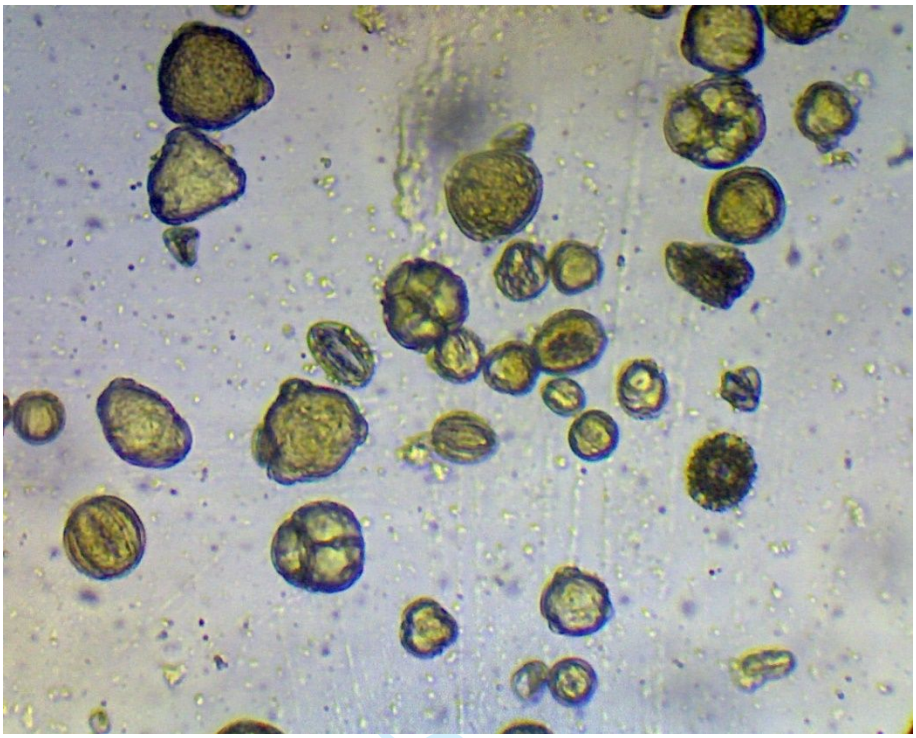


Fig. S6. Sample P06. Chestnut (*Castanea sativa*) dominant pollen type. Optical microscopy (magnification 100×).

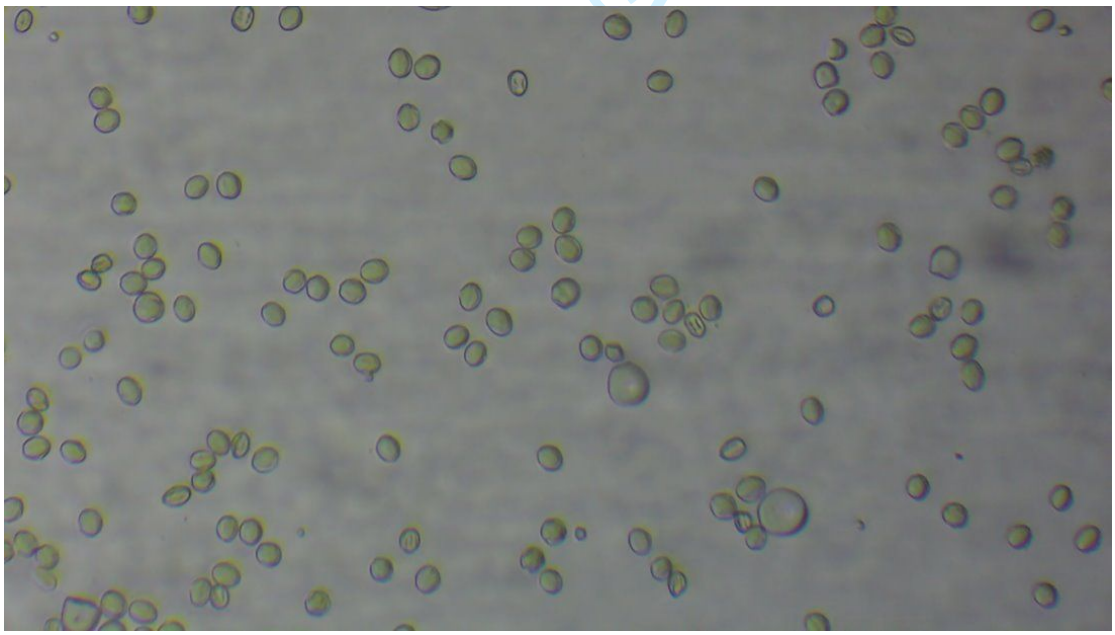


Fig. S7. Sample P07. Generic commercial pollen. Mixed pollen loads from plum tree pollen (*Prunus*, main pollen, upper panel) and from gum rockrose (*Cistus ladanifer*, lower panel) pollen. Optical microscopy (magnification 100×).

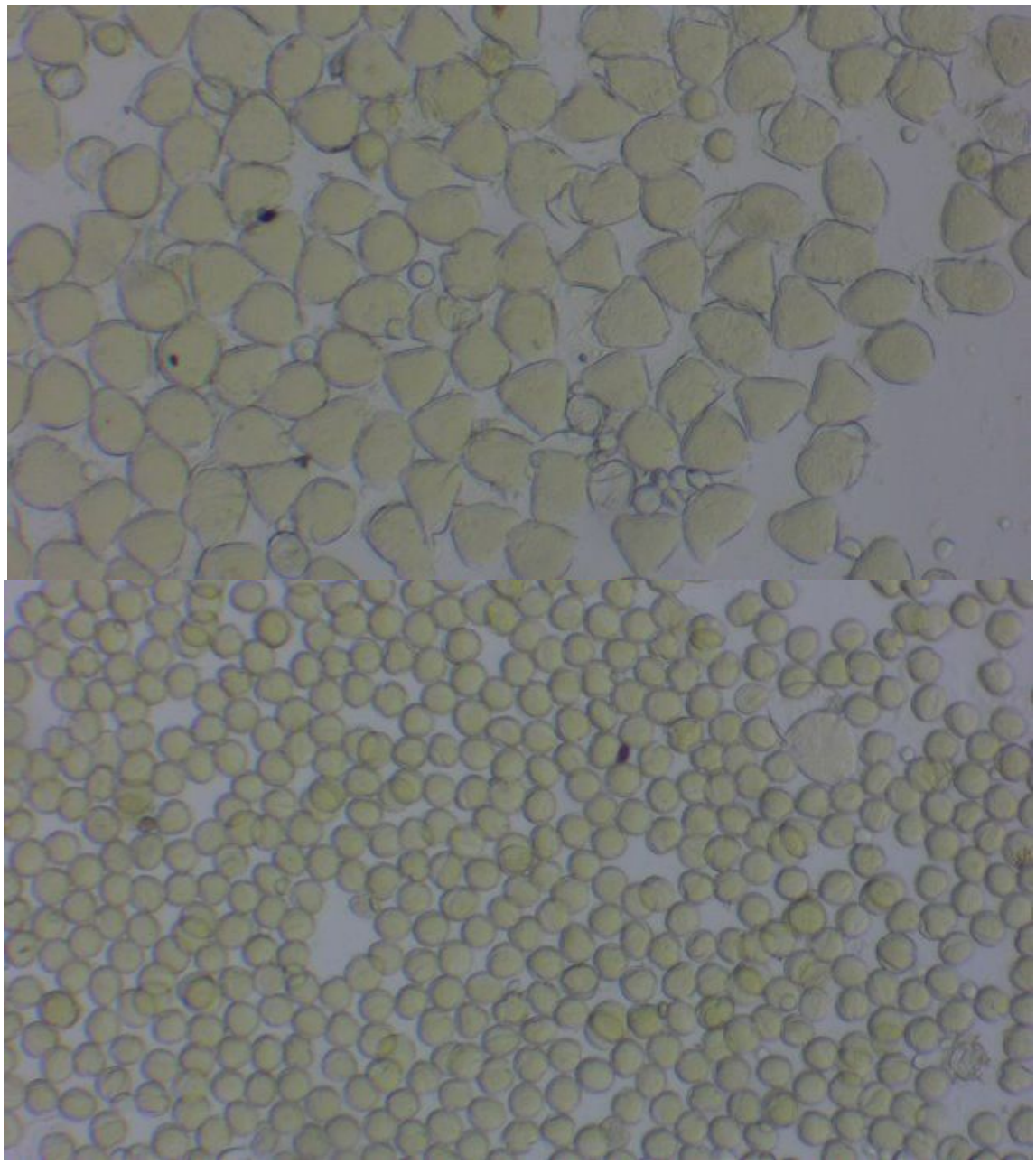


Fig. S8. Sample P08. Generic commercial pollen. Optical microscopy (magnification 100×).

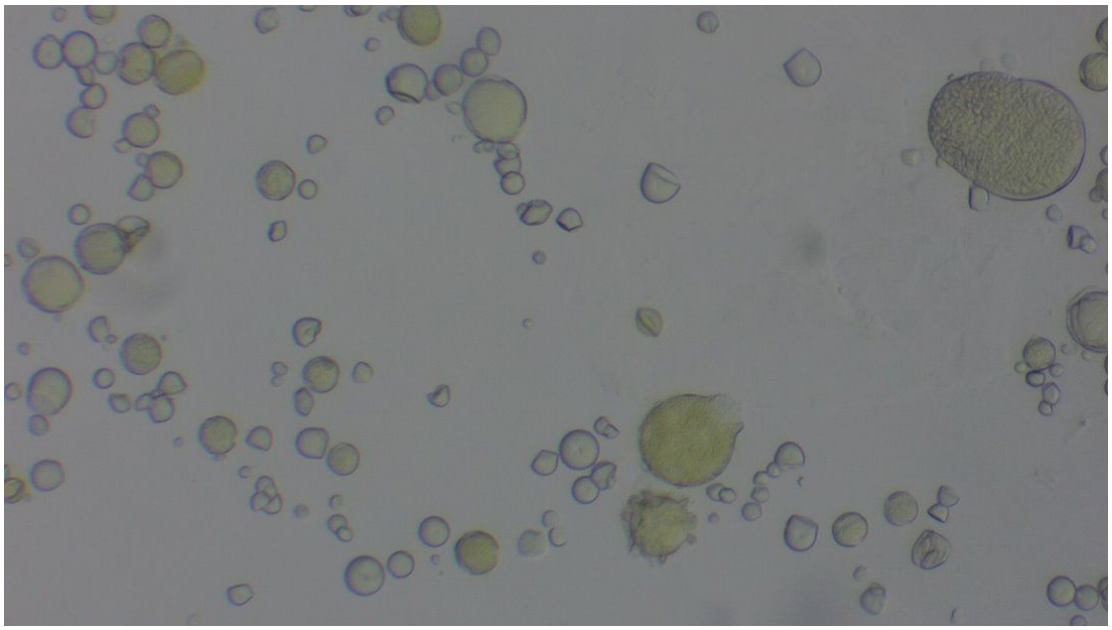


Fig. S9. Sample P09. Generic commercial pollen. Dominant pollen type from *Cistaceae*. Contrast phase microscopy (200×).

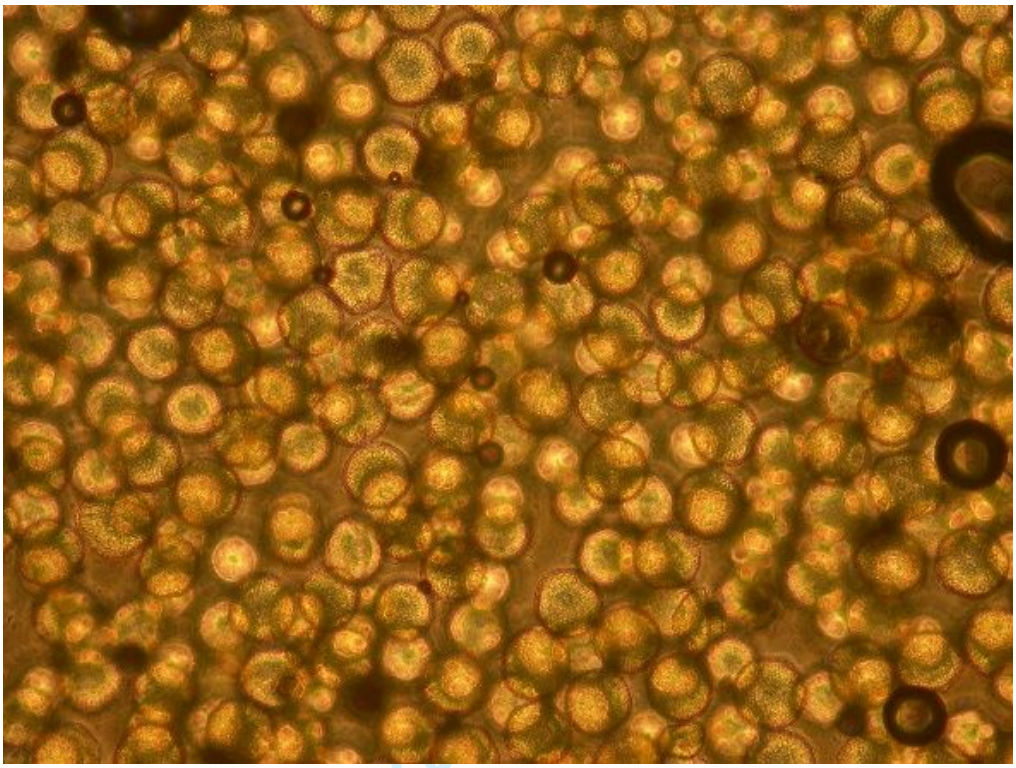


Fig. S10. Sample P10. Generic commercial pollen from Turkey. Mix of homogeneous pollen loads from *graminaceae* (upper panel) and a not identified pollen type (bottom panel). Optical microscopy (magnification 100×).

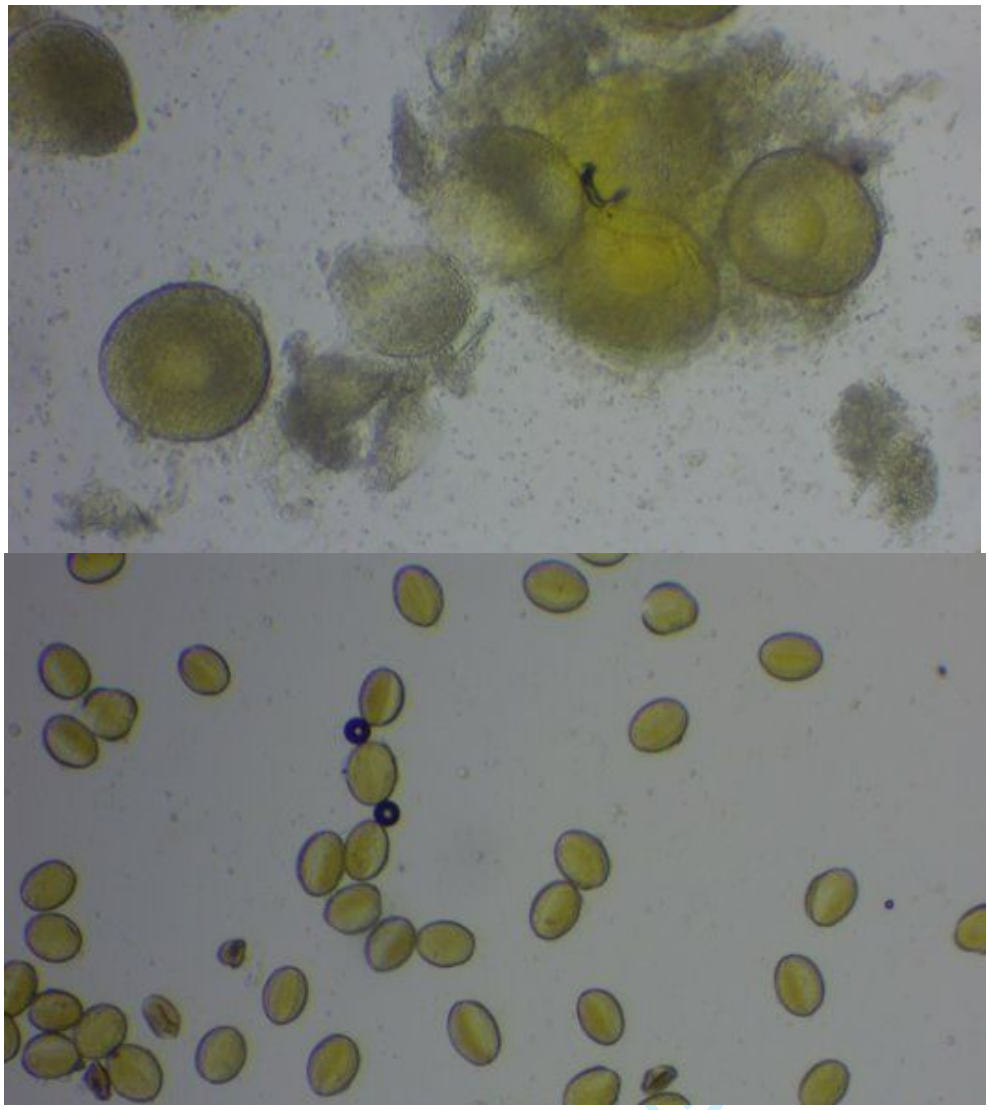


Fig. S11. Sample P11. Generic commercial pollen from a Kosovar beekeeper. Mix of pollen loads from not identified pollen types. Optical microscopy (magnification 100×).

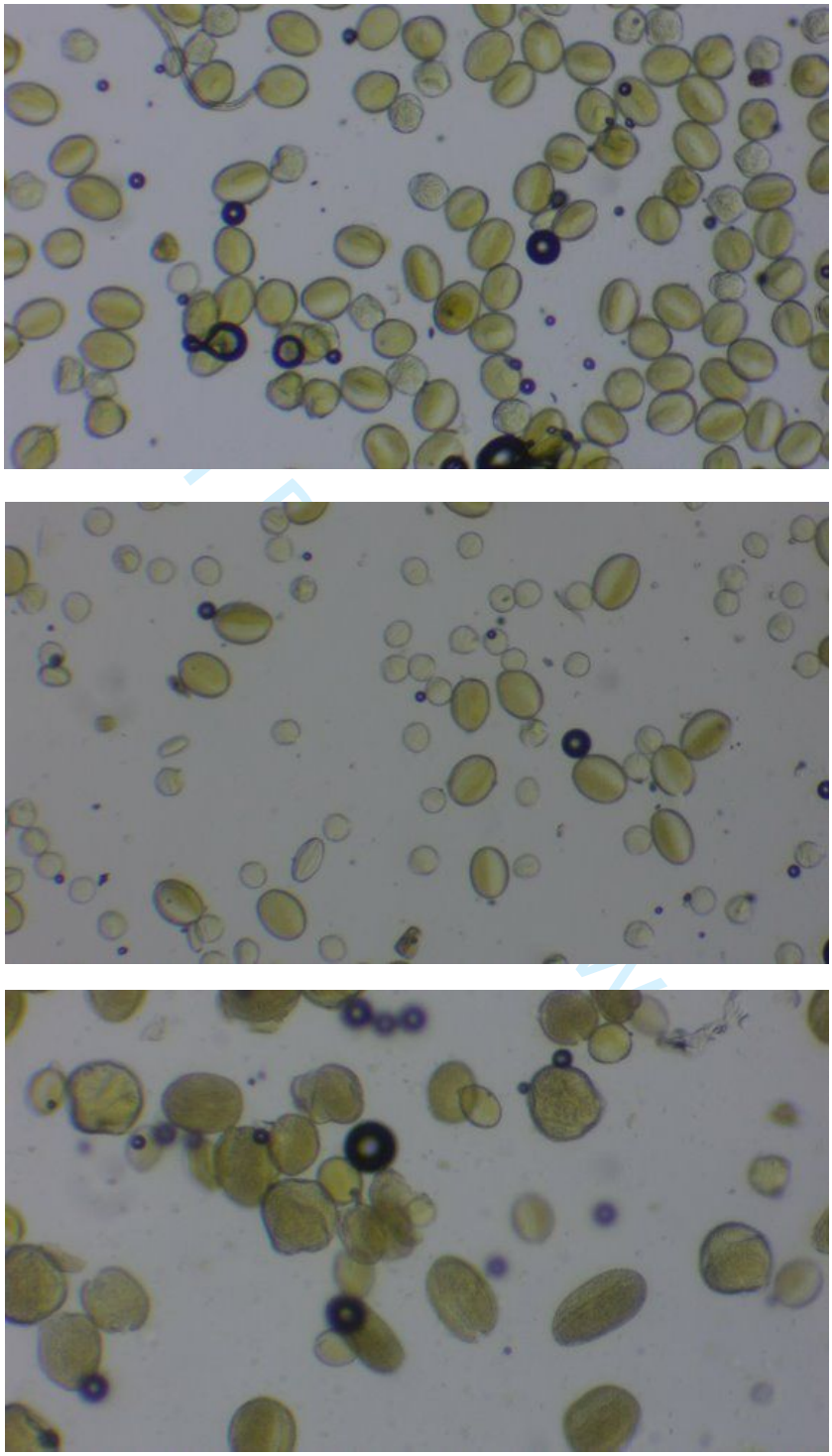


Fig. S12. Sample P12. Generic commercial pollen composed by dominant sunflower (*H. annuus*) pollen loads (Miracle Products, Kosovo). Optical microscopy (magnification 100×).

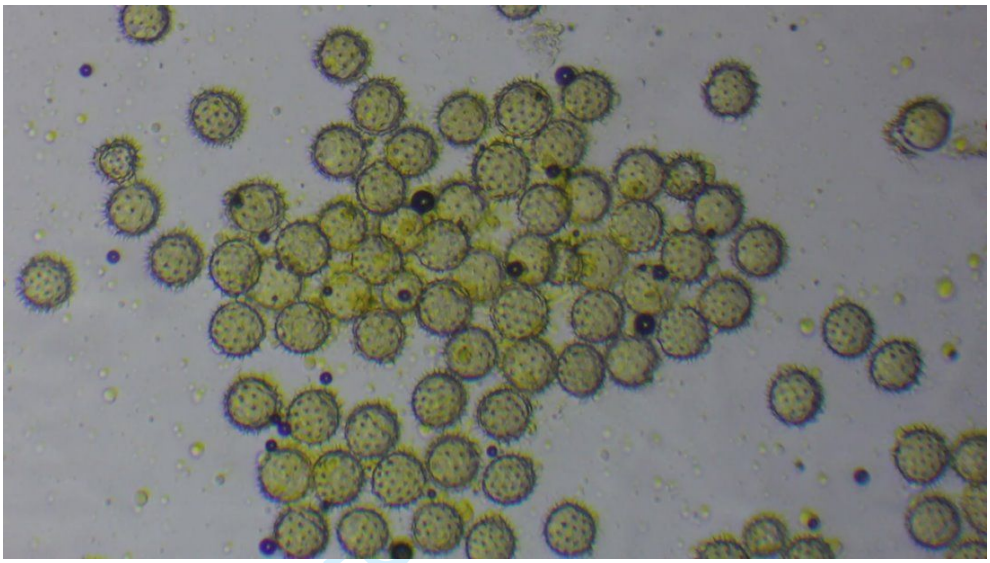


Fig. S13. Sample P13. (BIO HERBS, Kosovo). Meadow pollen types. Optical microscopy (magnification 100×).

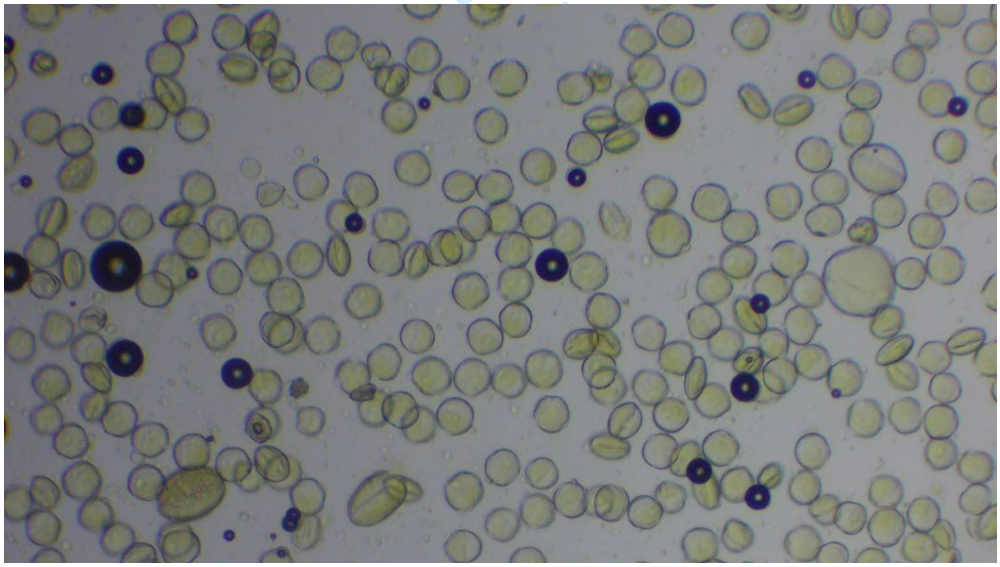


Fig. S14. Sample P14. (BIO HERBS, Kosovo). Pollen loads composition dominated by clover (*Trifolium repens* and *Trifolium spp).* Optical microscopy (magnification 100×).

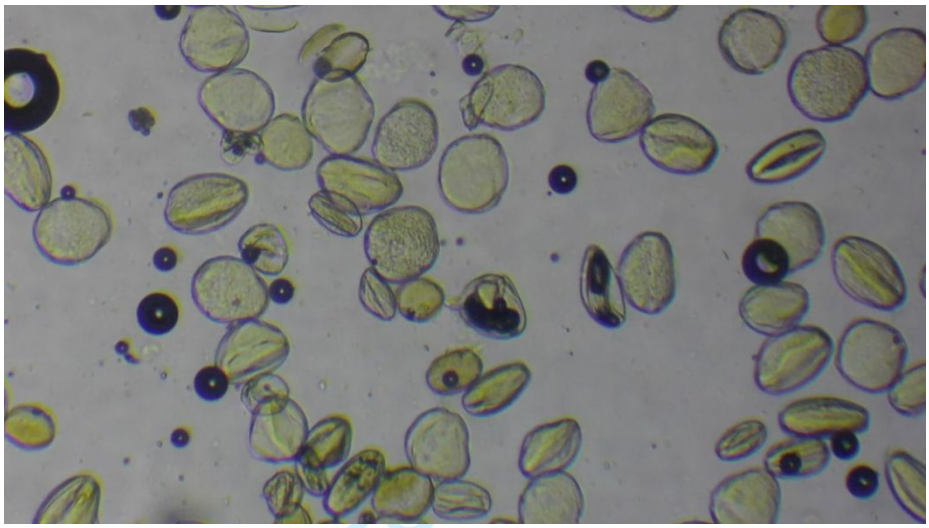


Fig. S15. Sample P15, generic pollen from a Kosovar beekeeper. Mainly composed by homogenous pollen loads: *graminaceace* (probale *Zea mais*; upper panel), Probable cow-wheat (*Melampyrum pratense*;) and sunflower (*H. annuus*; bottom panel). This main pollen types composition is suggestive of a agricultural production area. Optical microscopy (magnification 100×).

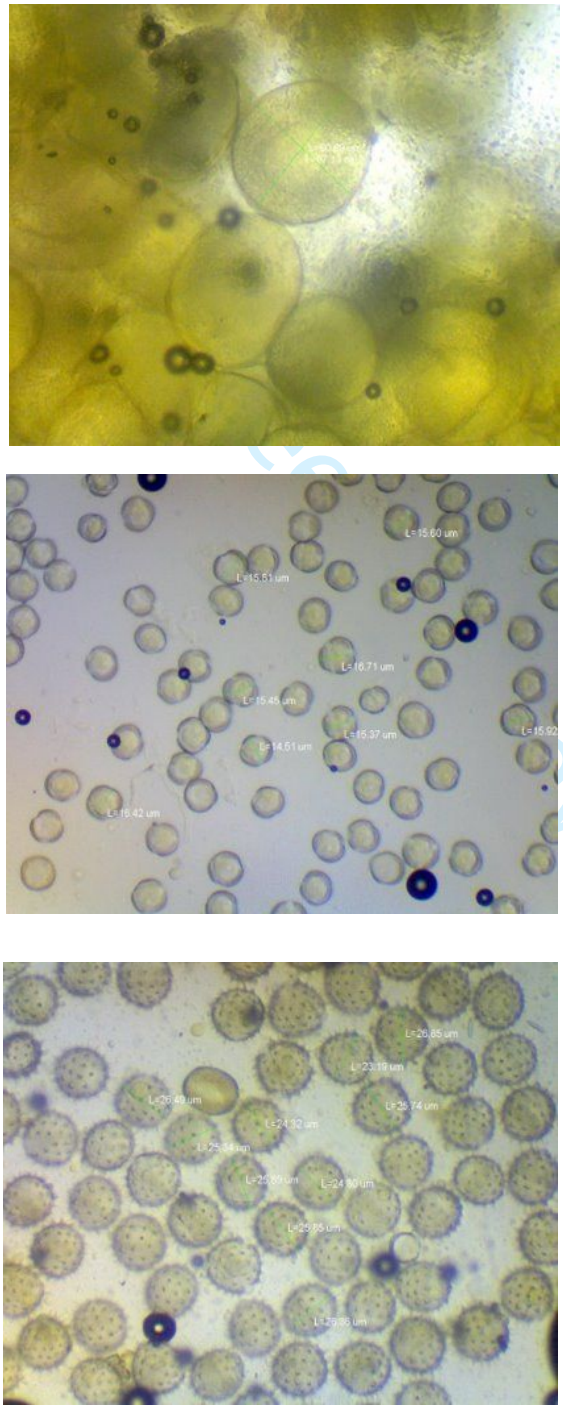


Fig. S16. Sample P16. (from beekeeper, Kosovo). Homogenous pollen loads composed by polles types originating probably from needle sunrose (*Fumana*; upper panel) and Solomon's-seal (*Polygonatum odoratum*; bottom panel) origin.

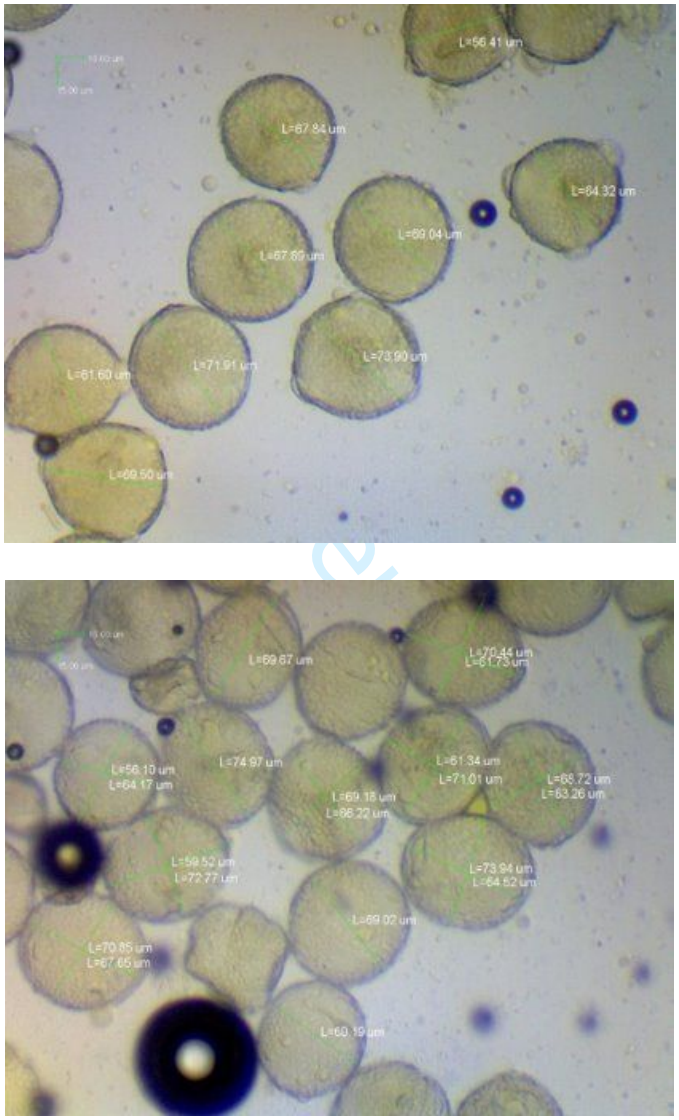


Fig. S17. Sample P17 from beekeeper of Bosna-Erzegovina. Homogenous pollen loads composed by polles types originating probably from probable Meadow buttercup (*Ranunculus acris*; upper panel), oat (*Avena sativa*); lower panel.

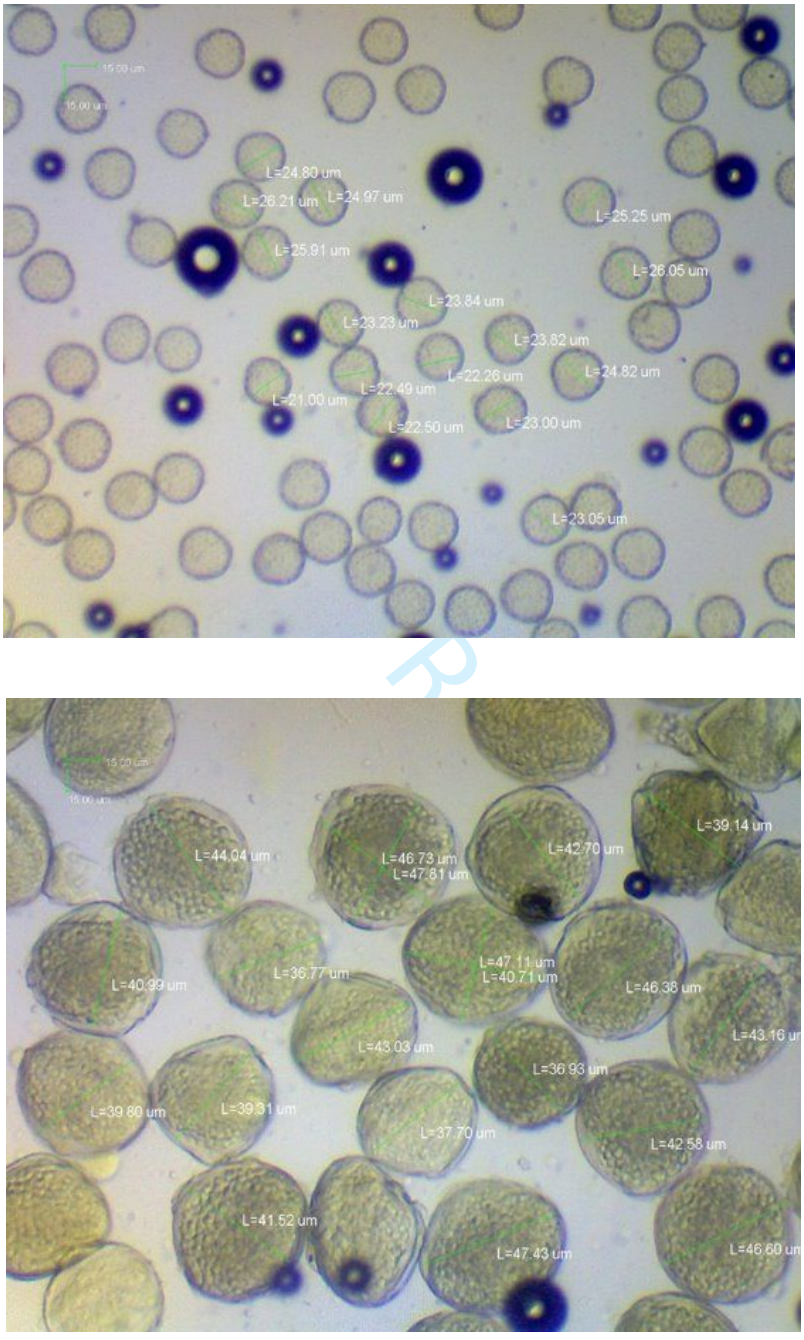


Fig. S18. Bee pollen ethanol extract yields

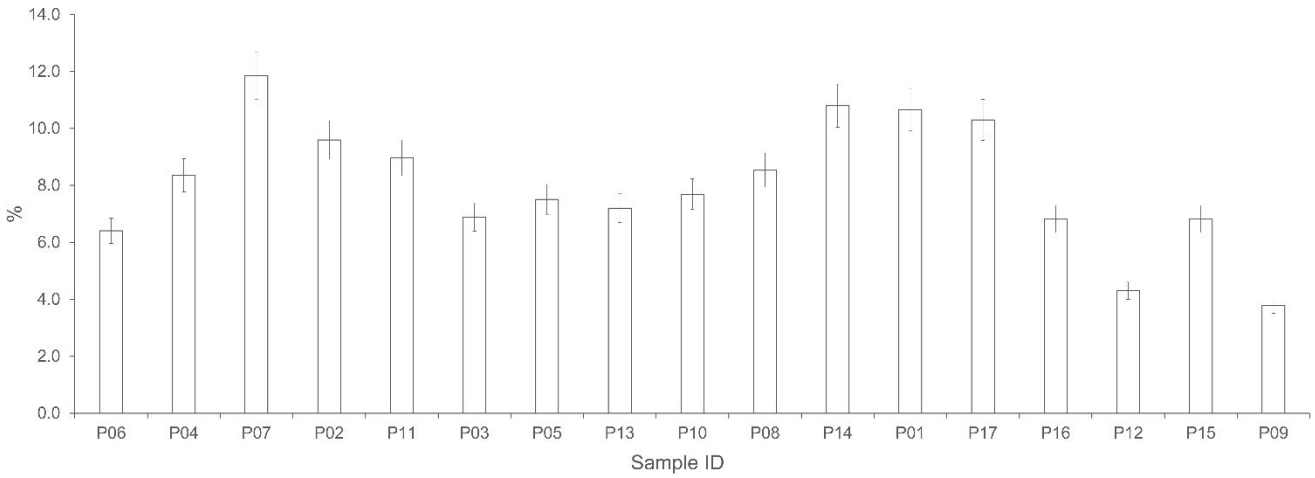


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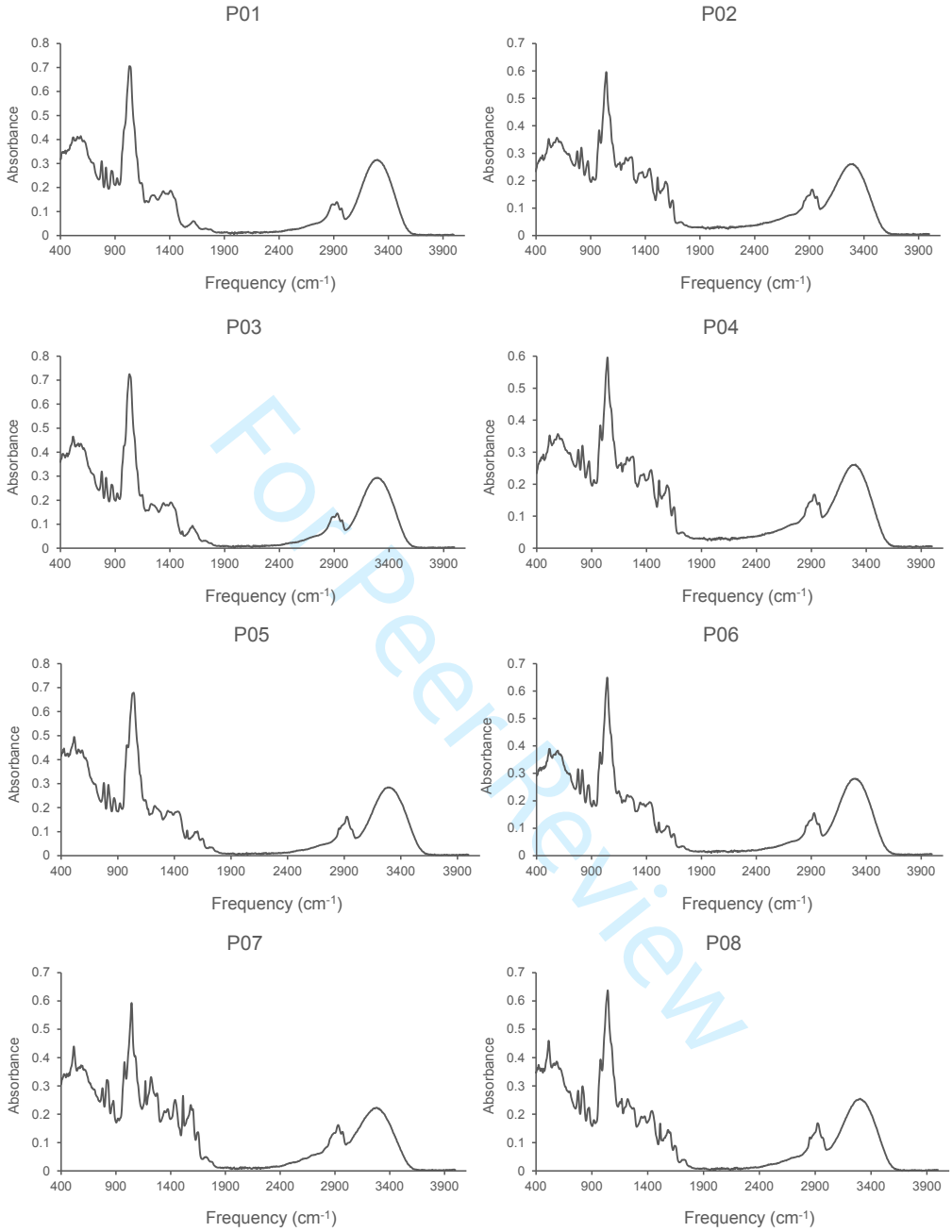
Fig. S19. ATR-FT-IR spectra of the of commercial pollen samples P01-P17 ethanol extracts.



(continues…)

(....follows)

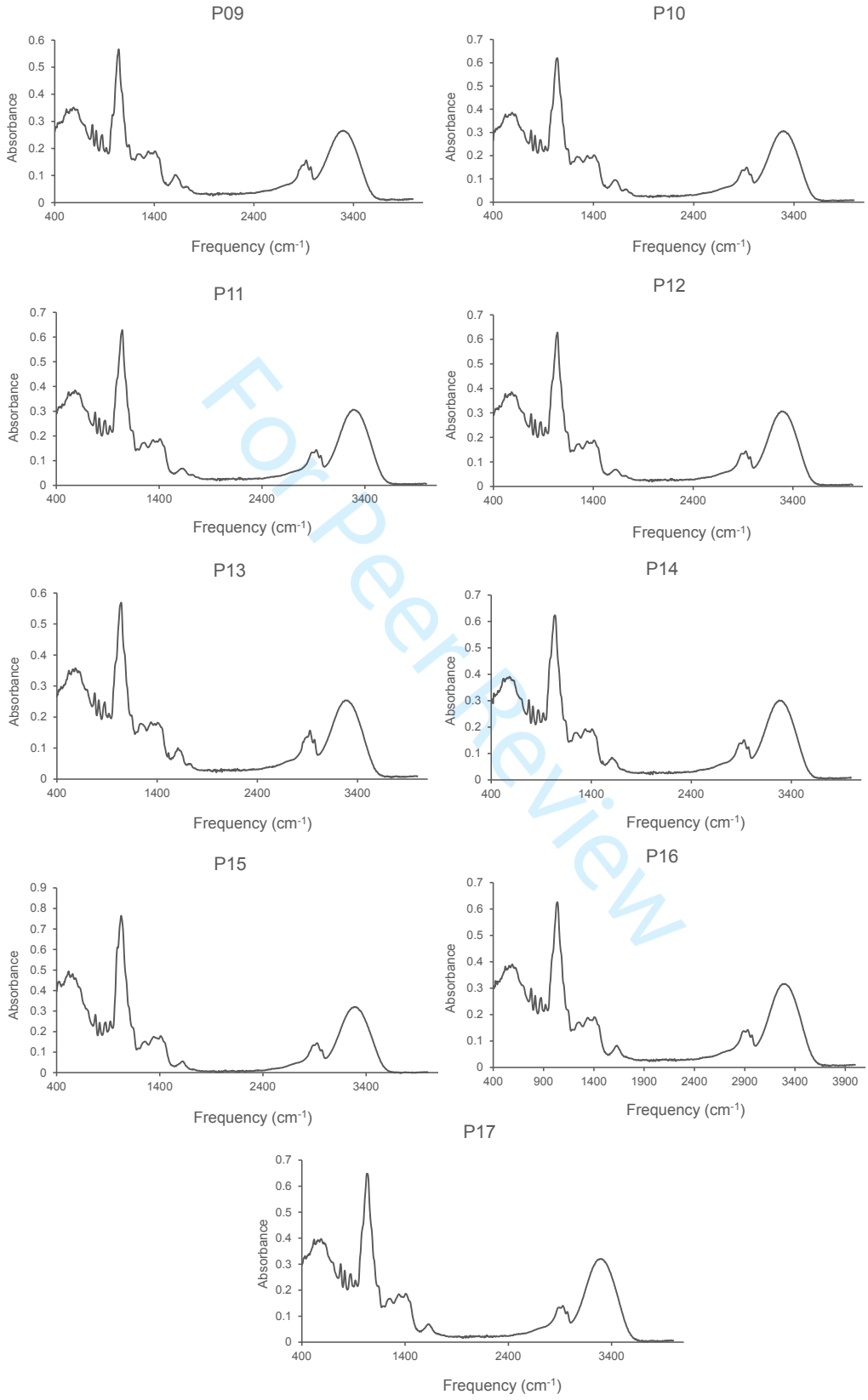
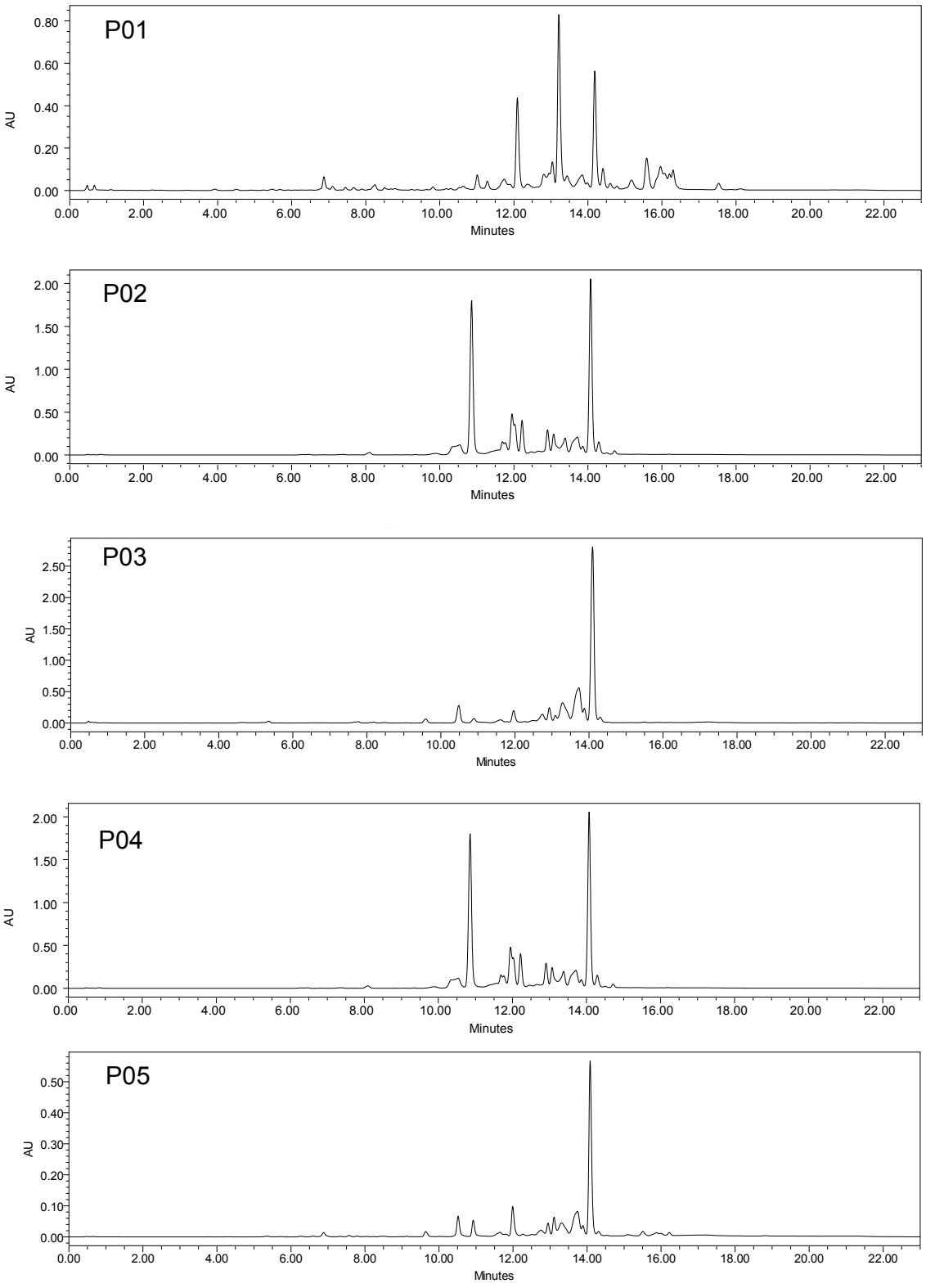
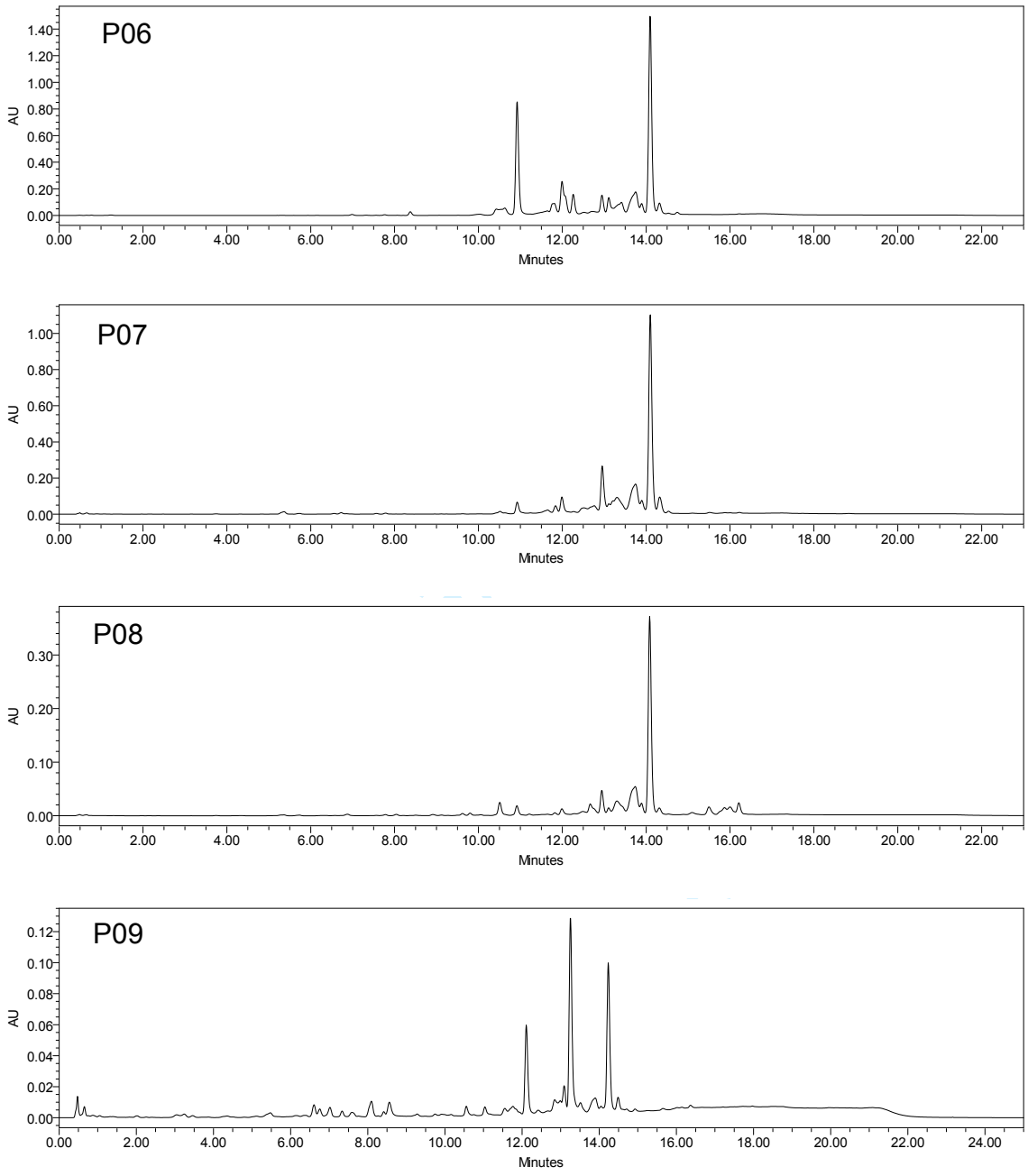


Fig. S20. UPLC®-PDA absorbtion profile of the commercial pollen samples P01-P17 ethanol extracts.



(continues…)

(…follows…)



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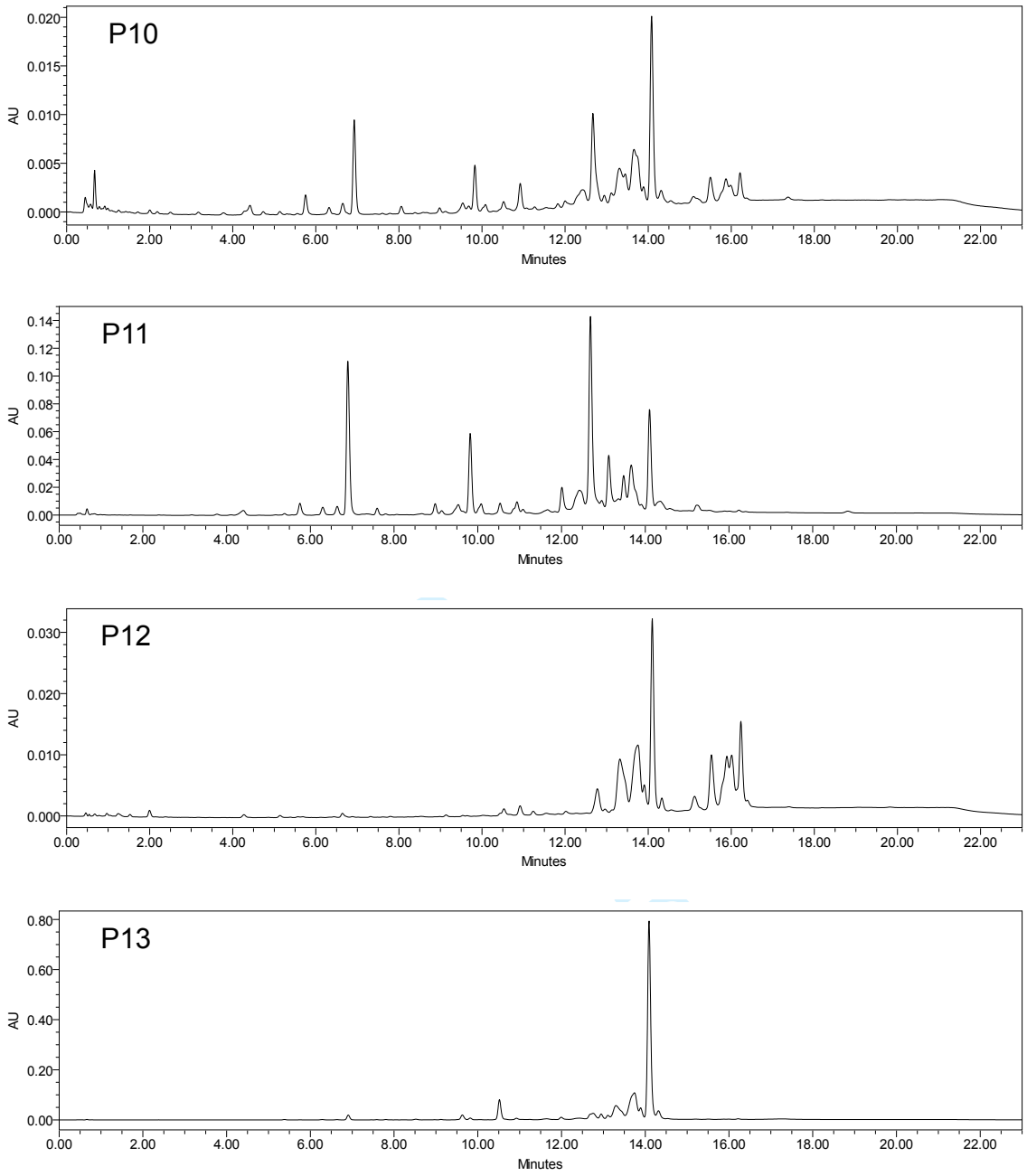
(…follows…)



(…continues…)

(...follows)

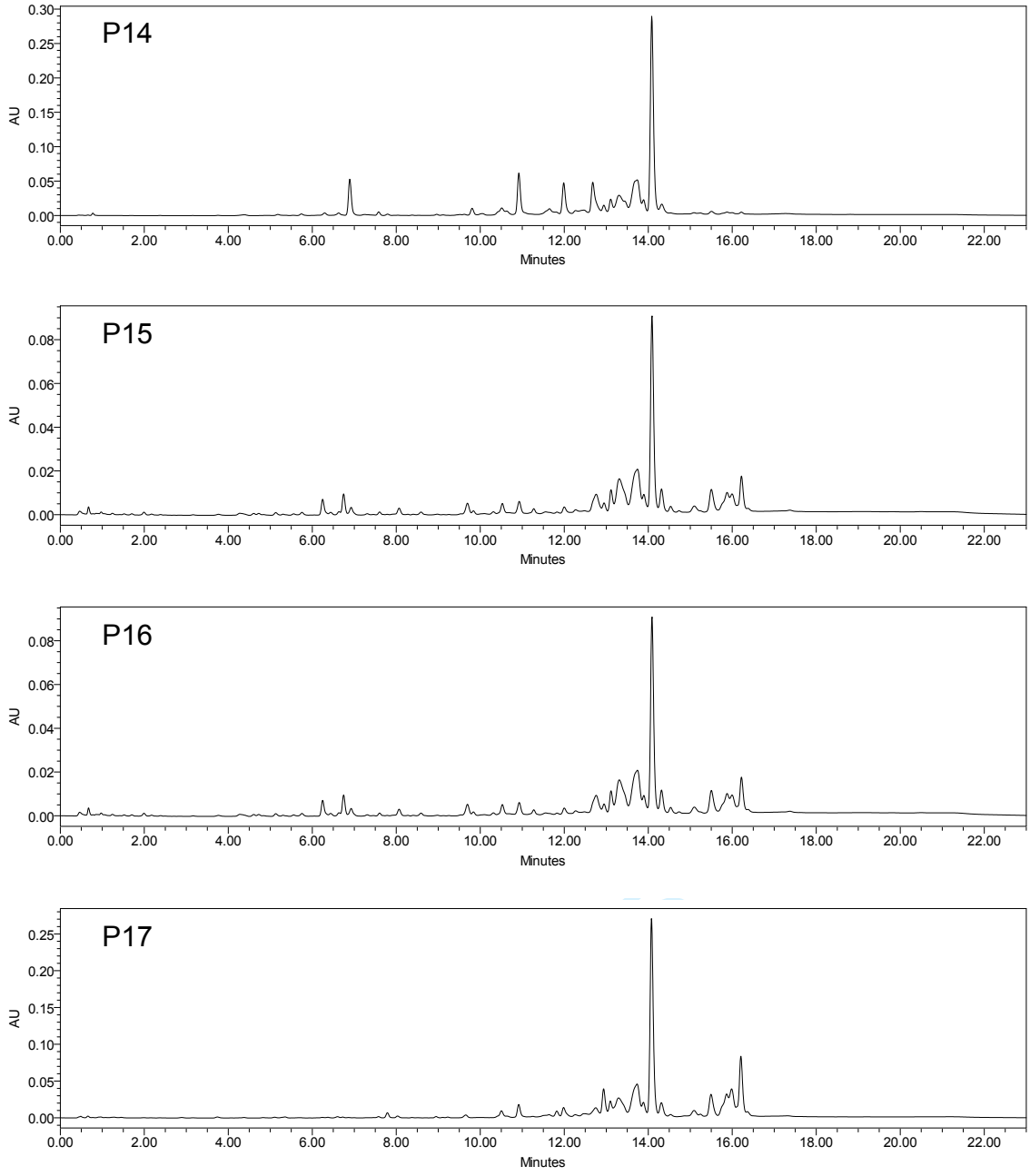
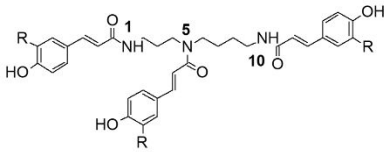
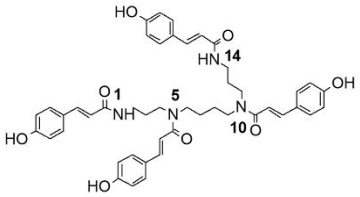


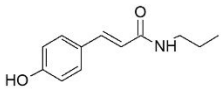
Fig. S21. identified isomers of HCPA N^1, N^5, N^{10}, N^{14}-tetra-substitued



1: N^1,N^5,N^{10}-tricaffeoyl spermidine (tri-C-Spdm)
2: N^1,N^{10}-di-caffeoyl, N^5-*p*-coumaroyl spermidine (di-C-Cu-Spdn)
3: N^1,N^{10}-di-*p*-coumaroyl, N^5-caffeoyl spermidine (C-di-Cu-Spdn)
4: N^1,N^5,N^{10}-tri-*p*-coumaroyl spermidine (tri-Cu-Spdn)

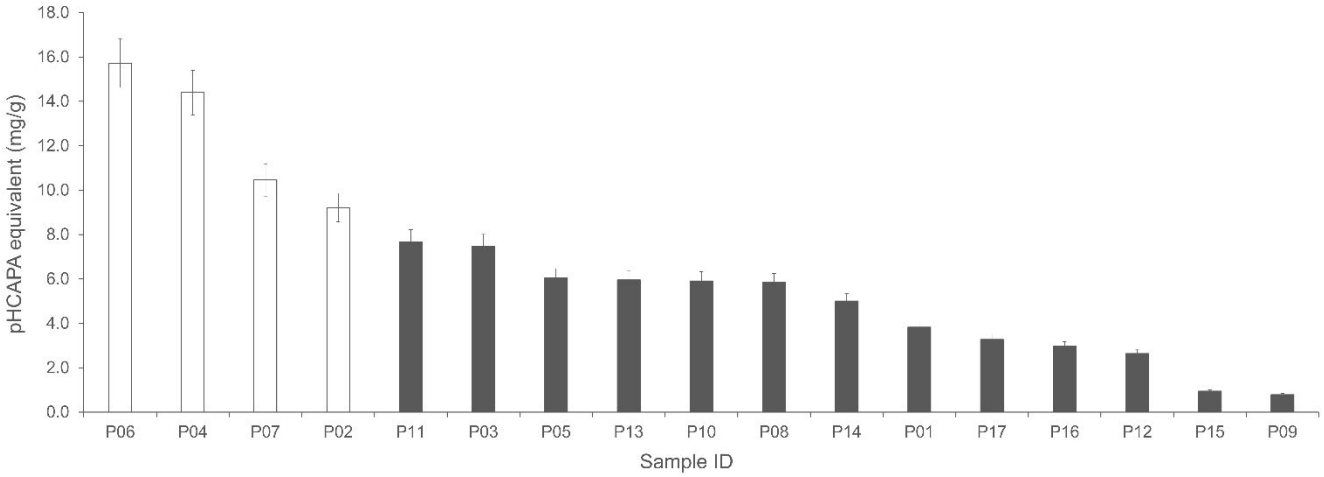


5: N^1,N^5,N^{10},N^{14}-tetra-*p*-coumaroyl spermidine (tetra-Cu-Spdn)



p-hydroxycinnamoyl propylamide (pHCAPA)

Fig. S22. Total HCPA concentration found in the samples P01-P17.



Synthesis of *p*-hydroxycinnamoyl propylamide (pHCAPA)

The synthesis of pHCAPA, (standard substance for quantitative analysis), was performed as previously reported by Fu et al. (2010), with minor modifications. Briefly, to a solution of *p*-hydroxycinnamic acid (1.22 mmol) in 2.5 mL of DMF and 0.17 mL (1.22 mmol) of trimethylamine cooled in an ice-water bath, propyl amine (1.22 mmol) was added followed by a solution of benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) (1.22 mmol) in 2.5 mL of dichloromethane. The mixture was stirred at 0 °C for 30 min and then left at room temperature overnight. The organic solvent was removed under reduced pressure, and the solution was diluted with 20 mL of water. The solution was extracted with ethyl acetate (3 x 5 mL), then washed with 1.0 M HCl, water, 1.0 M $NaHCO_3$ and water, dried over anhydrous Na_2SO_4, filtered and evaporated to give a crude solid residue. The product was purified on a silica gel column by flash chromatography eluting with a mixture of cyclohexane-ethyl acetate 1:1, affording the pure compound as a white solid (yield = 28.7%). Thin layer chromatography (TLC) retention factor (R_f) = 0.38 (stationary phase: silica gel; mobile phase: cyclohexane-ethyl acetate 3:7). Melting point (m.p.) = 141-143 °C. ^{1}H NMR (300 MHz, DMSO-d_6, ppm): δ 8.13 (t, J = 5.5 Hz, 1H, NH), 7.41 (d, J = 8.8 Hz, 2H, Har), 7.28 (d, J = 15.7 Hz, 1H, CH), 6.78 (d, J = 8.8 Hz, 2H, Har), 6.24 (d, J = 15.7 Hz, 1H, CH), 3.07 (td, J = 6.2, 5.5 Hz, 2H, CH2), 1.51 – 1.33 (m, 2H, CH_2), 0.82 (t, J = 7.4 Hz, 3H, CH_3).

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