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Reversed-phase liquid chromatographic characterization and analysis of air particulates humic (-like) substances in presence of pollens ☆

Róbert Góra, Milan Hutta *

Department of Analytical Chemistry, Faculty of Natural Sciences, Comenius University, Mlynská dolina CH-2, 842 15 Bratislava, Slovakia

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Abstract

Newly developed method for characterisation and analysis of humic substances (HS) and humic-like substances (HULIS) in air dust particles was tested for potential interferences caused by abundant co-sampled pollens (common dandelion, common wormwood-absinth, apple tree). RP-HPLC using 10-step gradient of dimethylformamide (DMF) in a buffered aqueous mobile phase and a wide-pore (30 nm) octadecylsilica column has been applied to the analysis of HS and HULIS using tandem of spectrophotometric (DAD) and fluorimetric detection (FLD). Achieved results suggest that the devised method is reliable for characterisation, fractionation and analysis of terrestrial HS, air dust HS and air particulate HULIS in liquid extracts at a trace concentration levels. Fluorimetric detection (ex. 470 nm/em. 530 nm; LOD, 3.1 μg/ml) enables sensitive, highly selective and interference free determination of HS and HULIS regardless the presence of pollen constituents, whereas spectrophotometric detection is susceptible to interferences in UV region above 260 nm. However, even in this case, the interfering substances can be revealed by both different pattern and shape of their peaks, as well as by spectral features different from HS or HULIS. Analytical procedure based on air sampling, extraction and above-mentioned HPLC method enables characterisation and analysis of HS and/or HULIS at relative concentration levels down to 0.1% (m/m) in 10 mg mass scale of sampled air dust and particulate material.
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Keywords: Air dust particles; Humic substances and HULIS analysis; Characterization; RP-HPLC; DAD; Fluorescence detection; Pollen interference

1. Introduction

Interest in analysis, determination or behaviour and structure elucidation of water humic substances (HS), including sedimentary HS and terrestrial (soil) HS is long lasting. However, relatively new focus is evident in analysis, characterization and fractionation of humic substances and humic-like substances in airborne particulate matter (HULIS) [1,2]; fog and interstitial aerosol [3]; characterization of water soluble organic matter from atmospheric aerosol [4,5] using solid-phase extraction procedures in a reversed-phase mode; estimation of the average relative molecular mass of HULIS isolated from fine atmospheric aerosol [6] alternatively by ultrafiltration and SEC; air particulates and aerosol HS and HULIS characterization by RP-HPLC using stepwise gradient elution by dimethylformamide in a ion-suppression mode [7]. However, during air dust and aerosol particles sampling we sometimes can not avoid also collection of pollens, because spores of fungi and pollens studied by aerobiology are the most abundant plant particles present in the air round the year throughout the globe and they are also transported by air (mainly pollens of anemophilic plants). Their quality and quantity varies with a plant cover, locality, climatic conditions and time period. Pollens have aerodynamic shape and diameter between 1 and 120 μm, majority of European common tree pollens have diameters between 15 and 35 μm, for the other allergenic plants it ranges from 30 to 50 μm.

Water extracts from urban airborne dust samples were analysed by capillary isotachophoresis by Sázelová et al. [8,9] for pollen allergens, organic pollutants and inorganic ions and recently liquid extracts from tree and grass pollens were analysed by capillary electromigration methods [10] using low UV photometric detection. These works confirmed very complex character of the allergenic extracts as

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* Corresponding author. Tel.: +421 2 602 96 307; fax: +421 2 602 96 706.
E-mail address: hutta@fns.uniba.sk (M. Hutta).

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was stated earlier [11,12]. Size-exclusion HPLC was used for relative molecular mass determination of proteinaceous substances of aqueous allergen extracts obtained from birch pollens, timothy pollens and mite by Wahl et al. [13,14].

Combined size-exclusion HPLC, isoelectrofocusing and radio-allergo-sorbent test were used by Bolzacchini et al. [15] for separation of several allergens from *Parietaria judaica* total extracts. Bolzacchini et al. [16] purified *Phleum pratense* grass pollen aqueous phosphate (pH 7.2) extracts by size-exclusion HPLC, immunoaffinity chromatography and high-performance ion-exchange chromatography and they got complex characteristic profiles at 280 nm wavelength photometric detection.

Detailed composition of a variety of pollens is not known yet, but some insight into its complexity can be obtained from a representative analysis of the composition of bee collected pollen [17]. The precise composition varies somewhat in dependence of the floral source and local soil types. Composition of dry pollen matter is 45% (m/m) proteins (proteins and glycoproteins), 40% (m/m) carbohydrates, 7% (m/m) fiber, 4% (m/m) lipids, 4% (m/m) ash (inorganics). Free amino acids alanine, arginine, aspartic acid, glutamic acid, glycine, histine, leucine/isoleucine, lysine, methionine, phenylalanine, proline/hydroxyproline, tyrosine, valine are present. Pollens contain vitamins riboflavin, nicotinic acid, pantothenic acid, pyridoxine, ascorbic acid, tocopherol, biotin, and at least 11 carotenoids, 8 flavonoids, the nucleic acids DNA and RNA, a wide variety of enzymes and a host of other components. Pollens also contain potassium, sodium, calcium, magnesium, phosphorous, sulfur, water, trace levels of aluminum, boron, chlorine, copper, iodine, iron, manganese, nickel, silicon, titanium and zinc [17].

RP-HPLC method devised by Hutta and Góra [7] proved to be well suited for the purpose of induction of distinct features in otherwise featureless analytical signal of air particulates humic substances. However, we feel that confirmation of validity of the devised method must be done also from the point of view of potential interferences of pollens as a probable source of potentially interfering substances co-sampled during surface station air sampling.

2. Experimental

2.1. Instrumentation

Study of the retention behaviour and evaluation of spectral characteristics of the selected groups of HULIS, HAs and compounds extracted from pollen samples was carried out by the HPLC system LaChrom (Merck-Hitachi, Darmstadt, Germany) consisting of pump L-7100 provided by a quarternary low-pressure gradient, autosampler L-7200, column oven L-7300, diode-array detector L-7450A, fluorescence detector L-7480, interface D-7000, PC data station with software HSM ver.3.1 and on-line four channel solvent degasser L-7612. Measured dwell volume of the system including column was 3.80 ml and should be considered when gradient mixing profile and chromatogram appearance is to be compared.

Extractions of HULIS from filters and air dust particulates were done with the aid of shaker KS 125 (IKA Labortechnik, Junke and Kunkel GmbH, Germany). Digital pH meter Radelkis OP-211/1 (Budapest, Hungary) provided by combined glass/AgCl electrode was used for mobile phase pH measurement.

Derenda and Purucker GS050/3 air sampler (Berlin, Germany), or Partisol 2000-H air sampler (Ruprecht & Patashnik Co., Albany, New York, USA) certified for the air particulates collection on filters were used alternatively for air dust or pollen collection.

2.2. Methods

Separation was carried out using a LiChroCART column 250 mm × 4 mm filled by wide-pore octadecylsilica LiChrospher WP 300 RP-18, 5 μm spherical particles, guarded by LiChroCART 4 mm × 4 mm pre-column filled by LiChrospher WP 300 RP-18, 5 μm particles. Dead volume (calculated from the manufacturers data [18]) of the column was 2.55 ml and is equal to the retention volume of the first HS eluted peak. Flow-rate was maintained at 1.00 ml/min.

Separation conditions for optimised gradient elution of humic substances and pollen extracts were as follows. Mobile phase A composition was: aqueous phosphate buffer (pH 3.00, 50 mM) containing 1% (v/v) dimethylformamide (DMF). Mobile phase B was 100% (v/v) DMF.

2.2.1. Stepwise gradient

Gradient program was set from 0.0 to 3.6 min isocratic 0% (v/v) B in A, and from 3.7 min, every 4 min there was isocratic step added increasing content of B in A by 10% (v/v) up to the last step increased by 9% (v/v) ending in 99% (v/v) B in A, maintained till 55.0 min isocratic 99% (v/v) B in A, from 55.1 min to 60.0 min linear decrease from 99% (v/v) B in A to 0% (v/v) B in A and between runs 10 min reequilibration was maintained [7].

Column oven temperature was maintained at $35.0 \pm 0.1\,^{\circ}C$. Injection volume of 50 μl was injected by the autosampler. Wavelength range of DAD was set to 260–800 nm. Monitoring wavelength was set to 280 nm. Fluorescence detection parameters were set to excitation wavelength 470 nm and emission wavelength 530 nm, photomultiplier gain medium was chosen.

Soil humic substances were isolated from soil sample collected at locality Dunajská Streda, Slovakia (FAO – Calcaric Fluvisoil, I. Horizon; depth, 10–20 cm) according to fractionation scheme described in detail by Procháckóvá et al. [19].

Air particulates (locality Topolové, Bratislava – an edge of meadow forest and field with arable soil) were collected at the quartz microfiber filter OM-A for air sampling (diameter 47 mm, Whatman) with the aid of Partisol 2000-H air sampler (Ruprecht & Patashnik Co., Albany, New York, USA). Sampled air volume was 110 m^3; air flowrate, 1.0 m^3/h). Mass of

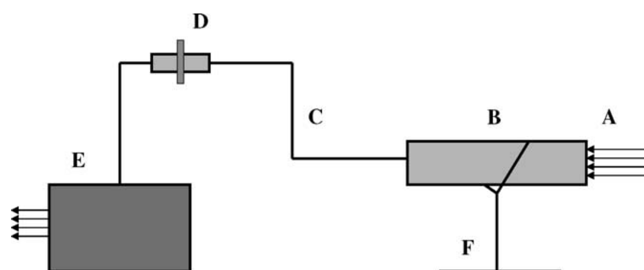

Fig. 1. Schema of Derenda and Purucker air sampler: (A) air inlet, (B) container polypropylene bottle with 1.5 l volume loosely packed by flowers collected for sampling of pollens, (C) sedimentation tube for removal of plant particles bigger than pollens, (D) holder of quartz paper filter and filter, provided by a 200 μm mesh inlet sieve, (E) Derenda and Purucker air sampler, (F) shaking arm.

sampled particulates (81.0 mg) was determined gravimetrically and corrected to moisture. One fourth of the filter (crosscut and corresponding to 20.25 mg of the particulates) was extracted succesively twice by 1 ml of 0.05 M NaOH in water for 2 h using laboratory shaker, so that 2 ml of the extract were obtained. The solution was then filtered through the same brand filter (previously purified by 0.05 M NaOH) and analysed in the HPLC system.

Pollens of various allergenic plants (common dandelion, common wormwood-absinth, apple tree) were collected at the quartz microfiber filter OM-A for air sampling (diameter, 47 mm; Whatman), with the aid of Derenda and Purucker GS050/3 air sampler (Berlin, Germany). The air sampler was provided by attached holder of sampled blossoming flowers of the plants mentioned above (see Fig. 1). Into this vessel freshly collected flowers of dandellion (300 pieces, Bratislava–Rača, May 2003), apple tree flowers (600 pieces, Bratislava–Rača, May 2003), common wormwood (Bratislava–Rača, August 2003) were loosely packed. Sampled air volume was 8–9 m^3, air flow-rate was 2.63 m^3/h, the container vessel was shaken with frequency 10 times per minute. Mass of air-dried sampled pollens was determined gravimetrically by microbalances Sartorius AWD 10, having sensitivity ±1 μg. Mass of the pollen matter ranged from 0.2 to 0.9 mg. Filter media were extracted successively twice by 1 ml of 0.05 M NaOH in water for 2 h using the laboratory shaker. The solution was then filtered through the same brand filter (previously purified by 0.05 M NaOH) and analysed in the HPLC system.

2.3. Materials

Working standard of solid humic substances was obtained by the procedures published by Procháckovà et al. [19].

Solutions of humic substances were prepared daily fresh by dissolution of weighed HS at 3.2 mg/ml concentration level in 0.05 M NaOH and were further diluted to required final concentration.

All runs were done using LiChroCART column 250 mm × 4 mm filled by a wide-pore octadecylsilica LiChrospher WP 300 RP-18, 5 μm spherical particles and guarded by LiChroCART 4 mm × 4 mm pre-column filled by LiChrospher WP 300 RP-18, 5 μm (Merck, Darmstadt, Germany). The column is devised mainly for fast reversed-phase analysis and preparation of peptides and proteins with the relative molecular mass up to 20 000 and tRNA molecules [18] without size-exclusion effects. The pore size of 30 nm allows an efficient mass transfer and a very good recovery, particularly of strong hydrophobic peptides. Its specific surface area is 80 m^2/g and pH stability is declared to be within the range 2.0–7.5.

Dimethylformamide DMF (Merck, Darmstadt, Germany, or Lachema, Brno, Czech Republic) was of pro analysi purity. Water for gradient HPLC was prepared by Labconco Pro-PS unit (Labconco, Kansas City, USA). Phosphoric acid (Lachema, Brno, Czech Republic) and sodium hydroxide (Merck, Darmstadt, Germany) of pro analysi grade were used for preparation of buffered mobile phases.

3. Results and discussion

3.1. Optimised separation conditions for profiling and characterisation of HULIS and HS

The devised stepwise gradient chromatographic method (10 steps) with tandem DAD and fluorimetric detection (FLD) [7] was used for characterization of HS, HULIS and dandelion pollen extracts by their chromatographic profiles. Fig. 2 shows typical examples of the background corrected profiles as resulted from analysis of humic acid HA Z (concentration, 50 and 10 μg/ml, respectively), APHLS B2 humic

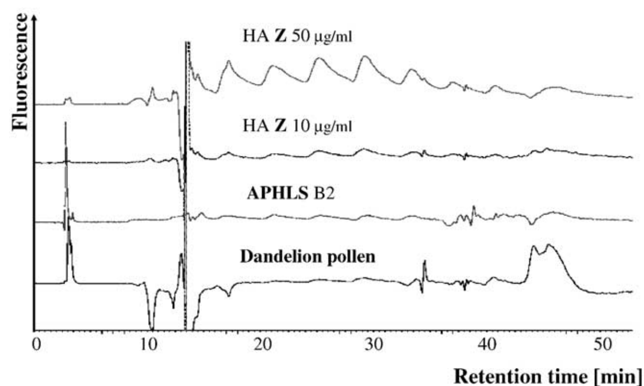

Fig. 2. Fluorescence chromatograms of dandelion pollen extract, air particulates humic-like substances extract APHLS B2, humic acid solution HA Z having concentration 10 μg/ml, HA Z 50 μg/ml (bottom-up) obtained by a stepwise gradient elution from a 30 nm wide-pore LiChrospher WP 300 RP-18 (5 μm, LiChroCART 250 mm × 4 mm, 4 mm × 4 mm pre-column) column by stepwise gradient elution. Mobile phase A composition: aqueous phosphate buffer (pH 3.00, 50 mM) containing 1% (v/v) of dimethylformamide (DMF). Mobile phase B was 100% (v/v) DMF. Flow-rate was 1.0 ml/min. Fluorescence detector set to excitation wavelength 470 nm, detection at emission wavelength 530 nm. Injected sample volume was 50 μl. Column oven temperature was 35.0 ± 0.1 °C.

substances and humic-like sbstances extracted from air particulates and dandelion pollen extract. Fluorescence detection gives chromatograms (Fig. 2) with distinctive features of humic HS's and HULIS enabling to differentiate among various HS and gives almost no signal of dandelion extract. Residual signal of dandelion extract similar to signal of HULIS can be probably attributed to dust particles co-sampled during preparation of pollen samples (mildly dusty flowers). Substances having low relative molecular mass were removed from humic acid HA Z during its preparation and purification by dialysis desalting (membrane with MWCO 8000). Humic acid HA Z was obtained [19] by dissolution/precipitation procedure after its release from the soil matrix by acid hydrolysis (6 M HCl). SEC profiles [20] of HA Z humic acid were resulting in the relative molecular mass ranged from 10 000 to 21 000.

Fig. 3 shows background corrected chromatographic profiles (detected at 280 nm) of the same dandelion pollen and humic-like substances extracted by NaOH solution from sampled pollen and air particulates, respectively. For comparison purpose also chromatograms obtained from soil humic acid HA Z were added, because the soil sample from which this HA was extracted resembles the soil and potentially air dust particles in the locality where air particulates were collected. At this flow-rate passage of the first gradient step through the detector cell is seen as a refraction error peak at 7.8 min at all chromatograms (refer to Figs. 2 and 3, respectively).

Chromatogram of the pollen extract gives (spectrophotometric detection at 280 nm wavelength) distinctively different features of discrete peaks in the retention time window from 24 to 30 min. Humic-like peaks eluted after 32 min are obvious and because they are eluted by 80% (v/v) and higher concentration of DMF in the aqueous buffer, we could attribute them to pollen waxes or the other non-polar polymeric material with aryl chromophores.

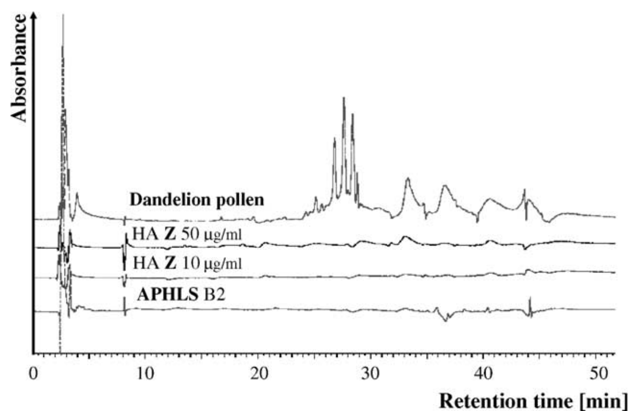

Fig. 3. Absorbance (UV) chromatograms of air particulates humic-like substances extract APHLS B2, humic acid solution HA Z having concentration 10 μg/ml, HA Z 50 μg/ml and dandelion pollen extract (bottom-up). Spectrophotometric detection (DAD) was done at monitoring wavelength 280 nm. Injected volume 50 μl. The other conditions and parameters are the same as in Fig. 2.

3.2. *Quantitative analysis of HS's and HULIS of air particulates*

Quantitative parameters of the method were tested during the investigation of humic-like substances isolated from air particulates (including soil dust particles). Recovery of the method as evaluated spectrophotometricaly ranged for the individual HS from 94% (m/v) to 102% (m/v) at concentration levels around 3 mg/ml. Attempts to determine or estimate concentration of humic-like substances were based on carefully chosen assumptions [7].

Repeatability of retention times of artificial peaks of HS was evaluated from the data obtained in seven consecutive runs of reference sample HA Z at the concentration level 3.2 mg/ml. Calculated repeatability of retention time (t_R) of selected peaks covers interval from ±0.14% to ±0.41% RSD. Repeatability of the data within one week (set of five data) was ±1.3% RSD. These data are representative also for the other well-shaped peaks of analysed substances, e.g., peaks of constituents of pollen extracts and confirm the values got earlier [7].

For quantitation of air particulate humic substances and HULIS, respectively, reference soil humic acid HA Z was chosen, because the soil type is similar to the soil in the area where the air particulates were sampled. For the fluorescence detection evaluated calibration curve was linear for the selected peaks with correlation coefficients of linear regression (least squares method) equal to 0.9941, the value which is comparable to values already published [7].

Quantitation of humic-like substances in air particulates was based on this individual calibration curve for peak having retention time 21.13 min with the regression equation $Y = 7.903 \times 10^{-5}X - 1.557$, where in area% method of the instrument software X is integrated peak area and Y is injected amount of HA; for details, see [7]. Further information on statistical treatment of the equation was not available, except the correlation coefficient equal to 0.9941.

This peak (eluting at 21.13 min) was selected according to criterion that it has almost the same relative peak area (13.7 ± 1.6%) for working standard HS Z, as has corresponding peak (retention time, 21.15 min) of HULIS in APHLS B2 in air particulates (13.9%). Relative peak area of HS Z was statistically evaluated from chromatograms measured at five concentration levels and was related to the total of the areas of all the components detected in the chromatogram (Area % method). Similarly we got the value for APHLS B2.

Determined concentration of HULIS was 9.5 μg/ml, what corresponds to the limit of quantitation (S/N = 10) of the method with fluorimetric detection. This corresponds roughly to 0.5 μg of HS per injection. Calculated limit of detection (S/N = 3, from chromatogram without background correction) was 3.1 μg/ml, what corresponds to 0.14 μg of HULIS in APHLS B2 per injection.

After recalculation to the humic-like substance concentration in the air particulates we got value 0.95 mg/g, what corresponds to relative mass concentration close to 0.1% (m/m),

however, expressed and interpreted as a concentration of similar soil humic acid (in our particular case HA Z), chosen arbitrarily as a working standard according to the defined rules [7].

3.3. Study of interferences caused by pollen extract constituents

During microscopic inspection of air sampled filters for air particulates we noted presence of various pollen grains, what led us to study their potential interference to HS's and HULIS analysis. As a test probe pollen we have chosen common dandelion pollen, because this plant is widespread medical plant which at our region blossoms since April till October (e.g., for Northeast USA the period is February–November) and is generally found in immediate or surrounding areas. It is a weed and is monitored by many local pollen reports and forecasts [21]. Dandelion pollen causes severe allergic reactions (polinosis) in people who are sensitive to other pollens such as ragweed.

Dandellion pollen grains are 45–48 μm in diameter, they have spheroidal shape with a triangular amb and long spines. Active compounds of the pollen are sesquiterpene lactones, triterpenes, vitamins A, B, C, D, coumarins, taraxacoside, phenolic acids, carotenoids, potassium and other inorganic substances. Many of these substances contain in their structures chromophores and some of them, or their chelates with metals could emit fluorescence (phenolic acids, flavonoids, etc.). We supposed that carotenoids could interfere in some cases, because they absorb light from 420 to 530 nm (maxima 460, 495, 525 nm). Fluorescence of carotenoids emitted at 535–560 nm can be measured at exciting wavelength 488 nm, what is close to the proposed detection conditions [7] of HS's (excitation at 470 nm, emission at 530 nm).

The other sampled test pollens were of apple tree and common wormwood-absinth. Last mentioned plants represented localised cultural plant (apple tree – pollen maximum in May) and main widespread allergy causing plant in our region (absinth – pollen maximum in August). All analysed pollen extracts showed similar chromatographic pattern as can be seen at Fig. 4 which presents zoomed chromatograms measured at 280 nm for two various dandelion pollen samples differing by collection period, collected pollen amount and locality. For comparison, apple tree pollen was analysed under the same conditions. Dandelion pollen sample II amount was 0.91 mg, dandelion pollen sample I amount was 0.33 mg, apple tree pollen 0.19 mg and they all were processed equally as the sample APHLS B2 (see under Section 2.2). Contrary to the typical wide HS's peaks induced by step gradient and present at the chromatogram (see, e.g., Fig. 2 for fluorescence detection) as one peak per gradient step, UV 280 nm chromatogram of pollen extracts contains regions with normal narrow chromatographic peaks. These peaks belong to pollen substances eluted from the wide-pore C18 silica column by 40% (v/v) and 50% (v/v) of DMF in aqueous phosphate buffer (pH 3.00), respectively. They are well resolved

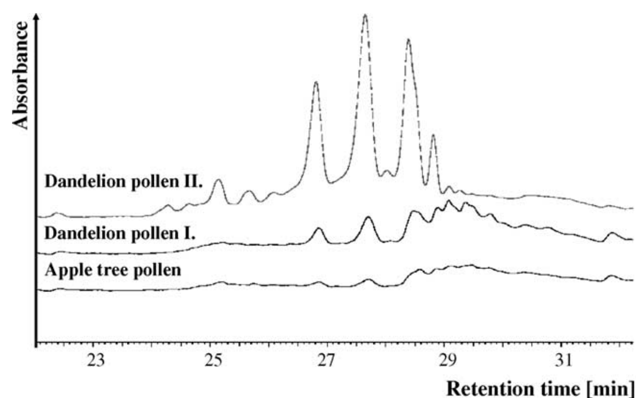

Fig. 4. Comparison of background corrected absorbance (280 nm) chromatographic profiles of various pollen extracts as obtained by stepwise gradient elution within the retention time range from 22 to 32 min. Extracts of dandelion I, dandelion II, and apple tree pollens, respectively, were prepared by the same extraction procedure. The other conditions and parameters are the same as in Fig. 3.

and within one gradient step representing elution by 40% (v/v) DMF we can count for dandelion pollen II sample at least 11 peaks (Fig. 4) instead of one potentially eluting peak of HAs. All chromatograms are of similar pattern, however, these sample constituents show no fluorescence under the fluorescence detection conditions devised for HS's and HULIS analysis (compare Figs. 2 and 3) and cause no problem of their quantitation.

According to Wahl et al. [13,14], birch pollen extracts contained fractions having relative molar mass around 8000, 14 000 and above 670 000, respectively. We suppose by analogy that even dandelion pollen substances having comparable relative molar masses can be chromatographed under given conditions without exclusion effects because the range of relative molar masses of restricted inclusion of the analytes for 30 nm pore diameter is relatively broad, ranging approximately from 4×10^4 to 2×10^6. Peptide or protein molecules with relative molar mass up to around 2×10^4 have not restricted access into the pores [18].

Method devised originaly for analysis of HS's, HULIS and lignins [7] can be modified also for the investigation and analysis of pollen constituents including beside low relative molar mass substances also proteins and glycoproteins. Aqueous and acidic extracts of birch pollen and *Dactylis* pollen, respectively, profiled recently by CZE and MEKC gave similarly low number of 8–15 major constituent peaks [10]. Pollen rich water extracts analysed by cITP [8] gave up to 20 distinguishable zones. However, discrimination power of cITP can be increased both for HS and pollen constituents by use of spacers as was done by Kopáček et al. [22] and Nagyová and Kaniansky [23] for HAs. Due to complexity of the problem of simultaneous analysis of HS's or HULIS and pollen constituents the discrimination power of the devised RP-HPLC method for next study should be increased by its combination either with cITP [24] or cITP-CZE [25,26].

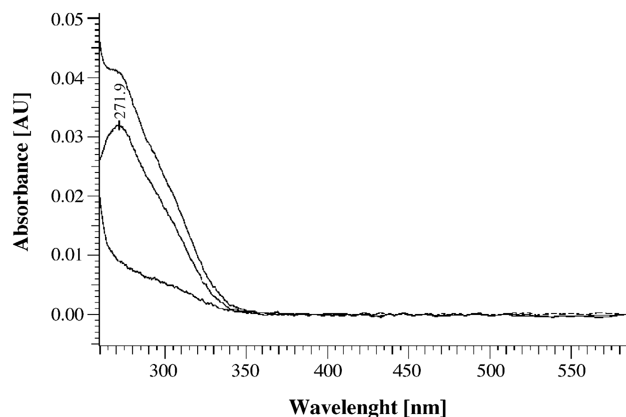

Fig. 5. Difference spectrum (background corrected, middle trace) of compounds eluted in 26.82 min from dandelion pollen II sample extract measured from 260 to 600 nm. Absorbance maximum of the peak is at 271.9 nm wavelength. For the conditions of chromatographic run, see Fig. 3. Lower trace is background spectrum, upper trace is non-corrected spectrum.

Spectra of two high peaks of dandelion pollen sample II extract eluted at defined retention times (26.82 and 27.65 min, respectively) are shown at Figs. 5 and 6. The spectra are similar and both have one absorbance maximum at 271.9 and 272.6 nm, respectively. They are different from UV–vis spectra of humic acids or spectra of artificial peaks of HS's induced by a stepwise gradient as is evident from Fig. 7, where for clarity spectrum of peak of HA Z eluted at 28.92 min is shown as a typical monotonous spectrum without extremes by featureless character similar to UV–vis spectra of HULIS [2–4,27] and HS's. Concentration of HA Z in the measured sample was 3.2 mg/ml, far above the concentrations practically needed for HS's and HULIS quantitation, which are 60–320 times lower. However, at these low concentration levels acquired spectra are too noisy at wavelenghts above 260 nm. Acquisition of spectra below 260 nm is not possible due to strong light absorption by DMF itself.

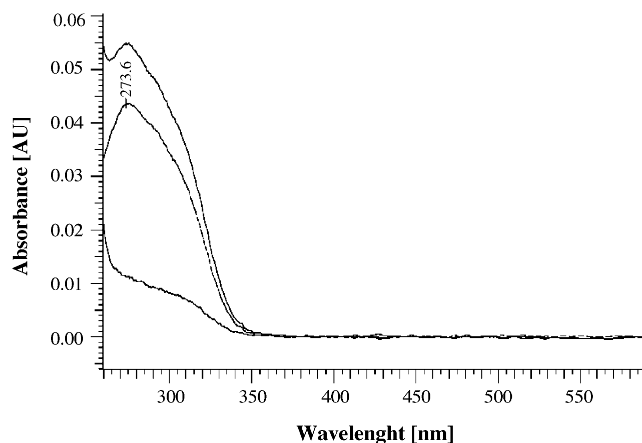

Fig. 6. Difference spectrum (background corrected, middle trace) of compounds eluted in 27.65 min from dandelion pollen II sample extract measured from 260 to 600 nm. Absorbance maximum of the peak is at 273.6 nm wavelength. For conditions of chromatographic run, see Fig. 3. Lower trace is background spectrum, upper trace is non-corrected spectrum.

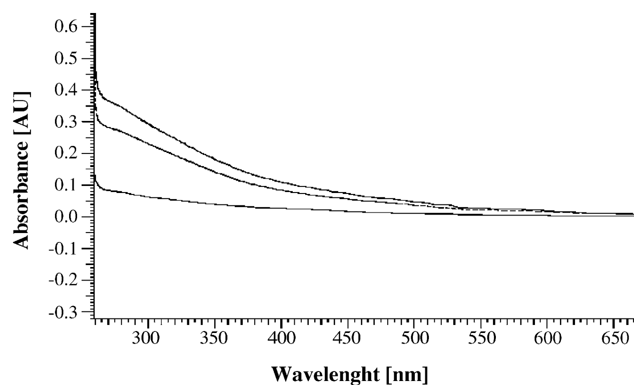

Fig. 7. Spectrum of humic acid HA Z (3.2 mg/ml) peak eluted at 28.92 min (refer to Fig. 3 but note that HA Z concentration is 64 and 320 times lower, respectively). Lower trace is background spectrum collected at 27.51 min, in the middle is different (background corrected) spectrum collected at 28.92 min, upper trace is actual spectrum collected at 28.92 min. For conditions of chromatographic run, see Fig. 3.

4. Conclusions

Analytical procedure based on above described air sampling, extraction and RP-HPLC method with fluorimetric detection enables characterisation and determination of HS and/or HULIS at relative concentration levels down to 0.1% (m/m) and their microanalysis in 10 mg mass scale of sampled air dust and particulate material.

Acknowledgements

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