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New study of anion-exchange chromatography for profiling of soil and peat humic substances and aromatic carboxylic acids

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Abstract

A simple anion-exchange liquid chromatographic method has been developed to assay commercial available humic substances, alkaline extracts of various types of soils and selected model aromatic acids. The anion-exchange HPLC analysis used short glass column (30x3 mm) filled with Separon HEMA-BIO 1000 sorbent with (diethylamino)ethyl functional groups (60 μm particles) and a mobile phase A constituted of aqueous solution of tetrasodium salt of EDTA (pH 12.0; 5 mmol L^{-1}) and mobile phase B constituted of aqueous solution of tetrasodium salt of EDTA (pH 12.0, 500 mmol L^{-1}). The wavelength of the detection is 480 nm (λ_{ex}) and 530 nm (λ_{em}) for humic substances and soils and 280 nm for aromatic acids. The proposed method provided an accurate and precise analysis of commercial available humic substances, alkaline extracts of various types of soils and selected aromatic acids. It was experimentally proved that under the selected conditions model aromatic acids are separated according to increasing effective negative charge and by analogy we suppose the same separation model also for fulvic acids and humic acids.

Keywords:

Anion-exchange chromatography; EDTA; humic substances; soils; aromatic acids

1. Introduction

The importance of separation methods in the chemistry of humic substances (HS), including liquid chromatographic (LC) methods is currently stressed by a review article of Hutta et al [1] that reviews advance of the topic since the review of Janoš [2]. From the literature results follows that recently the most frequently used separation methods for analysis and characterization of humic acids (HA) and fulvic acids (FA) are column LC methods (mainly SEC, less RP-HPLC, and their combination HPLC-SEC) [3-5] and electroseparation methods [6-8]. Hutta *et.al.* [9] showed the usefulness of stepwise gradient elution in RP-HPLC for characterization of HSs and lignins as a natural precursors of HSs.

In spite of direct evidence for potential of ion-exchange mechanisms, there is lack of articles on this topic. Majority of information related to the ionic properties of humic substances and/or ion-exchange separation mechanisms can be found in literature sources dealing with natural or waste water treatment by use of ion-exchange membranes and their fouling by HSs [10, 11]. Therefore we decided to evaluate analytical potential of the application of stepwise gradient [9] in combination with anion-exchange chromatography (IEC) for analysis and characterization of various types of samples in alkaline medium. Based

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on preliminary study [12] we came to the conclusion that very acceptable is research focus to the study of behavior od HA, FA and model aromatic carboxylic acids in alkaline media which is by definition of humic substances operational medium coming in the very first contact with humic substances.

EDTA (2,2',2",2"'-(ethane-1,2-diyldinitrilo)tetraacetic acid) is widely used to dissolve various inorganic salts including lime, substance accompanying humic substances in certain soil types, hence we decided to study potential of this compound as the sole buffering component of mobile phase at pH values high as 12.0. This pH was selected by a reference to the alkaline environment used for extraction of soil organic matter. Also at this pH high ionization of the functional groups (mainly carboxylic and phenolic) of HSs and their partial or complete disaggregation are expected. The usefulness of EDTA as eluting agent is evident because in alkaline medium it can bear high net charge up to the value of four minus commonly as tetrasodium or tetrapotassium salt [13], thus creating conditions of relatively high ionic strength at relatively low concentration in the mobile phase. Under selected conditions its enables to reach ionic strengths high as 5 mol L^{-1} (0.5 mol L^{-1} Na_4EDTA), thereby offers with a strong elution power also for the most bonded HSs. Since being a hexadentate ligand EDTA also acts as a competitive chelating agent, i.e. in the mobile phase it has an ability to "sequester" metal ions such as Ca^{2+}, Al^{3+} and Fe^{3+} present frequently in soil samples containing humic substances. After being bound by EDTA, metal ions remain in solution but exhibit diminished reactivity. This feature of EDTA probably supports the disaggregation of the aggregates of HSs, however this hypothesis must be proved.

We report the verification of separation mechanism in development of a simple IEC assay with diode array UV (DAD) and fluorimetric (FLD) detection for the analysis and characterization of commercially available HAs, various types of organic matter extracted by alkali solutions from soil samples and several model aromatic acids representing various negative charge characteristics.

2. Experimental

2.1 Chemicals and reagents

All the reagents and model analytes were of analytical-reagent grade unless stated otherwise. Water for gradient HPLC was prepared by Labconco Pro-PS unit (Labconco, Kansas City, USA). Sodium hydroxide (Merck, Darmstadt, Germany) was used for preparation of solutions of humic substances, soils and aromatic acids. Superpure EDTA (Sigma Aldrich, Steinheim, Germany) of was used for preparation of mobile phases. All aromatic acids (benzenecarboxylic acid, 2-hydroxy-benzenecarboxylic acid, benzene-1,2-dicarboxylic acid, benzene-1,4-dicarboxylic acid, 2,6-dihydroxy-benzenecarboxylic acid, benzene-1,2,4 tricarboxylic acid, benzene-1,2,5-tricarboxylic acid and benzene-1,2,4,5-tetracarboxylic acid) and hydroxybenzene were purchased from Sigma-Aldrich.

Commercial humic acid was purchased from Sigma-Aldrich (denoted as ALD) with relative molecular mass 500-1000, according to product catalogue. Humic substance Ecohum (denoted as ECO) was a commercially available fertilizer isolated form peat with ammonia solution at industrial scale. Humic substances Cerová and Suchá Hora (denoted as CER and SH, respectively) were isolated from peat using fractionation procedure recommended by IHSS [14, 15]. The soil samples used in this study were collected from different localities of Slovakia (Gbely, Šajdikové Humence, Senec and Kremnica-Skalka; denoted as GBELY, ŠAJDÍK, SENEC, SKALKA, respectively), whereby their pedological characteristics are summarized in Table 1.

Table 1. Pedological characteristics of selected soil samples according to [16].

Locality	Sampling depth (cm)	pH H_2O	pH KCl	Content of HS (%)	Type of soil
Šajdíkove Humence	0-10	3.96	2.83	16.9	umbrisol
Kremnica-Skalka	0-10	3.95	3.72	26.4	andosol
Senec	0-10	7.93	7.26	1.72	chernozem
Gbely	0-10	6.57	5.77	5.17	smonica

2.2 Instrumentation

Study of retention behavior and selected groups of HS and soils was carried out by the HPLC system LaChrom (Merck-Hitachi, Darmstadt, Germany). Measured dwell volume of the system including column with DAD/FLD tandem detector combination was 2.08 mL and should be considered when gradient mixing profile and chromatogram appearance is to be compared. An inoLab pH 730 (WTW, Weilheim, Germany) pH meter with combined glass/argentochloride electrode was used throughout, whereby all measurements of pH were carried out with correction to the alkaline error. Extractions of HSs from soils and peat were done by the laboratory shaker KS 125 (IKA Labortechnik, Kunkel, Germany).

2.3 Methods

A glass column (30x3 mm) filled with 0.25 g of Separon HEMA-BIO 1000 (diethylamino)ethyl (DEAE) sorbent (60 μm particles, Tessek, Prague, Czech Republic) was used for the fractionation of HSs, alkaline soil extract and model organic acids. This type of macroreticular hydroxyethyl-methacrylate ion-exchanger is feasible for separation of biomacromolecules with the relative molecular weights up to 1000 kDa [17-19], therefore this sorbent should allow the separation of HSs including those with the largest declared molecular weight (at the level of 500 kDa) without size exclusion effects. The selected resin offers with 0,8-1,2 mmol g^{-1} anion exchange capacity. According to the manufacturer information the main advantage of HEMA sorbents is in possibility of their in column sanitation by 0.1 mol L^{-1} NaOH as required by FDA protocols [20]. This statement and practical experience [12, 20-22] approves its possibility to use in alkaline medium. The DEAE group has pK_a value of 11,5, hence under separation conditions (pH 12.0) every functional group of the resin holds effective charge of 0.27. Dead volume of the prepared columns was fall between the range of 0.35-0.47 mL and is equal to the retention volume of the HS eluted in the first peak.

The separation conditions for optimal gradient elution of HSs were as follows. Mobile phase A composition was: aqueous solution of sodium salt of EDTA (pH 12.0, 5 mmol L^{-1}). Mobile phase B composition was: aqueous solution of sodium salt of EDTA (pH 12.0, 500 mmol L^{-1}). Gradient program for fractionation of HSs, extracted soil organic matter and separation of organic acids (used as model compounds) was set from 0.0 to 1.9 min isocratic 0% B in A than from 2.0 to 3.9 min isocratic 1% B in A and from 4.0 min every 2 min there was isocratic step added by increasing of the content of B in A by a factor of 2 up to the last step increased by 36% ending in 100% B in A, maintained till 30.0 min isocratic 100% B in A, from 30.1 to 33.0 min linear decrease from 100% B in A to 0% B in A and between runs 5 min re-equilibration was maintained. It must be stressed that this gradient produced distorted peaks of aromatic acids. The optimal gradient for separation of organic acids was a modification of the proposed stepwise gradient by an extension of all gradient steps from 2 min to 3 min, while the rest of the solvent mixin program remains the same as the previous program.

Table 2. Chromatographic conditions

Column	30x3 mm, Separon HEMA-BIO 1000 DEAE 60 μm
Wavelength	λ_{ex} 480 nm and λ_{em} 530 nm for standard HS and soil extracts; 280 nm for aromatic carboxylic acids
Injection volume	25 μl for standard HS and soil extracts and 10 μl for aromatic carboxylic acids
Flow rate	1.0 mL min^{-1}
Column temperature	40°C
Run time	38 min (with 2 minute gradient step duration) 47 min (with 3 minute gradient step duration)

2.4 Mobile phase buffer preparation and

Buffer solution B (500 mmol L^{-1} EDTA; pH 12) was prepared by dissolving 146,12 g of EDTA in the approximately 800 mL of water followed by adjusting of the pH of the solution to 12.0 with saturated solution of NaOH and finally made up the volume to 1000 mL. The buffer solution A (5 mmol L^{-1} EDTA; pH 12) was prepared by dilution of buffer solution B. It can be noted that the long term storage of the buffer solution B should be avoided, due to its high affinity to atmospheric CO_2.

2.5 Sample Preparation

The stock solutions of HSs were prepared daily fresh by dissolution of 3 mg of HS per 1 ml of 0.01 mol L^{-1} NaOH. The extraction procedure was performed in the following way: 1 g of sieved soil sample (15 mesh) was treated with 10 mL of 1.0 mol L^{-1} NaOH solution for 30 minutes. The solid residue was discarded. Prior to analysis the stock solutions and the soil extracts were centrifugated for 15 minutes at 3500 rpm. Samples of aromatic acids were prepared by dissolution of weighed aromatic acids at 1.00 mg mL^{-1} concentration levels in 0.01 mol L^{-1} NaOH (concentration of benzoic acid was 5.00 mg mL^{-1}).

3. Results and discussion

3.1. Adaptation of the stepwise gradient elution

Humic substances are characterized by high polydispersity of almost all properties, therefore the authors focused to the selection of such a gradient shape which could create distinguishable profiles of HSs in a similar fashion as is created in the RP-HPLC assay [4, 9]. The authors stressed, that the step-wise gradient elution mode could be used for distinct features gaining also in anion-exchange chromatography.

Initial attempts of fractionation of HSs were made using a column (30x3 mm) packed with strongly basic anion exchanger (HEMA BIO 1000 Q, quaternary ammonium functional groups, 60 μm particles). However the results have shown that the retention of the HSs on this type of anion exchange resin is high make impossible their complete elution even using 0.5 mol L^{-1} Na_4EDTA solution. After this experience we decided to study the impact of reducing the effective positive charge of ion exchange resin on the retention behavior of HSs. For this purpose we have substituted the strongly basic anion exchanger with moderately basic, containing DEAE functional groups. This substitution has led to the significant increase of the chromatographic yield of HSs, hence for fractionation of HSs the DEAE functionalized resin was used throughout.

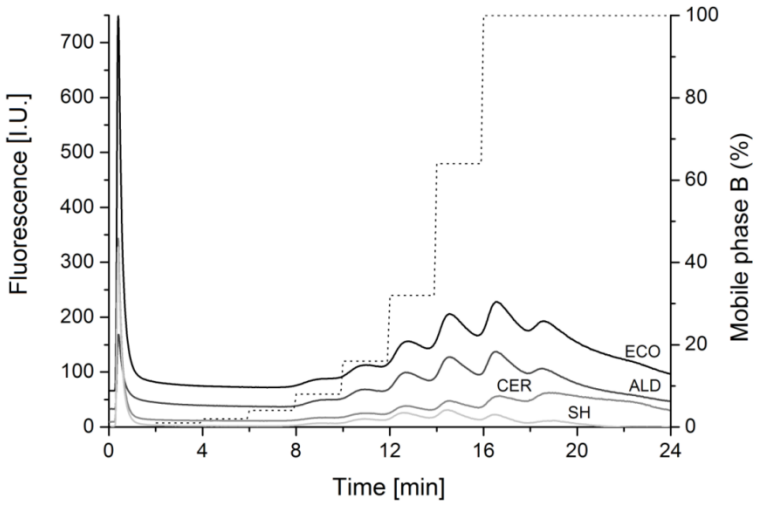

Fig. 1. Chromatographic profiles of selected humic substances (HA Cerova denoted as CER, HA Aldrich denoted as ALD, HA Ecohum denoted as ECO and HA Sucha Hora denoted as SH). For chromatographic conditions see Table 2. Scheme and shape of used stepwise gradient is illustrated on the figure.

Chromatograms of HSs profiling are shown in Figure 1 and they clearly demonstrate ability of devised system to generate characteristic peak-like profiles of HAs of various origin-location as-well-as alkali extracts of soils collected in various localities in Slovakia extracted by 1 mol L^{-1} NaOH containing corresponding HSs (Figure 2). It could be stressed, that the observed profiles are enforced by the step gradient shape. The Figure 1 shows nine-step gradient shape beside the obtained chromatograms. Except the first step, start of every step is overlaps with the valley between two peaks. Thereby, the highest peak is in the profile of ECO, ALD, CER and SH is eluted by 327, 327, 500 and 163 mmol L^{-1} solution of Na_4EDTA, respectively (64, 64, 100 and 32% of mobile phase B in A, respectively). These values are calculated with respect to the dwell volume of the chromatographic system.

From the figures also follows, that the proposed chromatographic method is capable to distinguish between the HSs from various origin analogous to the method described in [4] which was based on the hydrophobic interactions. Whereas the profiles of the "standard" HSs are similar the profiles of the alkaline extracts of the soils from localities Gbely and Senec differ in profiles from those of localities Kremnica-Skalka and Šajdíkové Humence. The main observed difference is in the proportion of signals attributed to non-retained or weakly retained HSs (of yellow color – beside fluorescence also absorbing light at 420 nm) eluted by 5 mmol L^{-1} $EDTA^{4-}$ and medium to strongly retained HSs eluted successively by mobile phase containing between 50 mmol L^{-1} and 500 mmol L^{-1} $EDTA^{4-}$ at pH 12.0. Applying the analogy between the types of the analysed soils with the obtained profiles we can conclude, that this method can probably differentiate between non- or weakly retained HAs and strongly retained FAs. This speculation needs further prove by analyzing well-characterized HSs with defined content of HAs and FAs. Still, these aspects are outside of the scope of this article and will be published elsewhere.

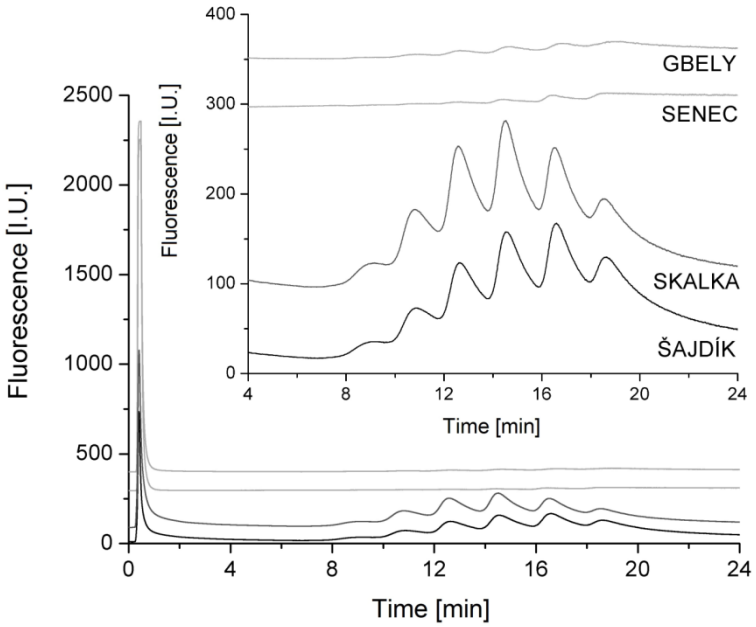

Fig.2. Chromatographic profiles of humic substances in extracts of soils from locations of Gbely, Šajdikové Humence, Senec and Kremnica-Skalka (all in Slovak Republic). For chromatographic conditions see Table 2.

3.2. Verification of the separation mechanism

Apparently separation principle is based on the ionic interactions and the fractions of the HSs are eluted by their increasing net charge. However this presumption needs to be verified. To elucidate separation mechanism from the point-of-view of effective charge of humic substances we studied on the base of analogy the properly chosen set of aromatic acids representing net charges 1-, 2-, 3- and 4-. Representatives of the charge 1- at the pH 12.0 are phenol, benzoic acid; charge 2- salicylic acid, phthalic and terephthalic acid; charge 3- are 2,6-dihydroxybenzoic acid, benzene-1,2,4 tricarboxylic acid and benzene-1,2,5-tricarboxylic acid; charge 4- is benzene-1,2,4,5-tetracarboxylic acid, were injected to the separation system under the identical conditions as described for the profiling of the HSs.

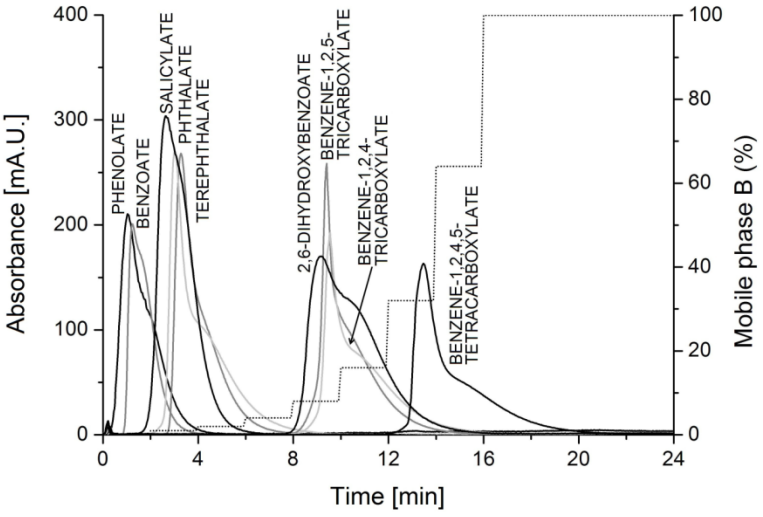

Fig 3. Chromatographic profiles of selected aromatic acids using stepwise gradient with 2 minutes steps. For chromatographic conditions see Table 2. Scheme and shape of used stepwise gradient is illustrated on the figure.

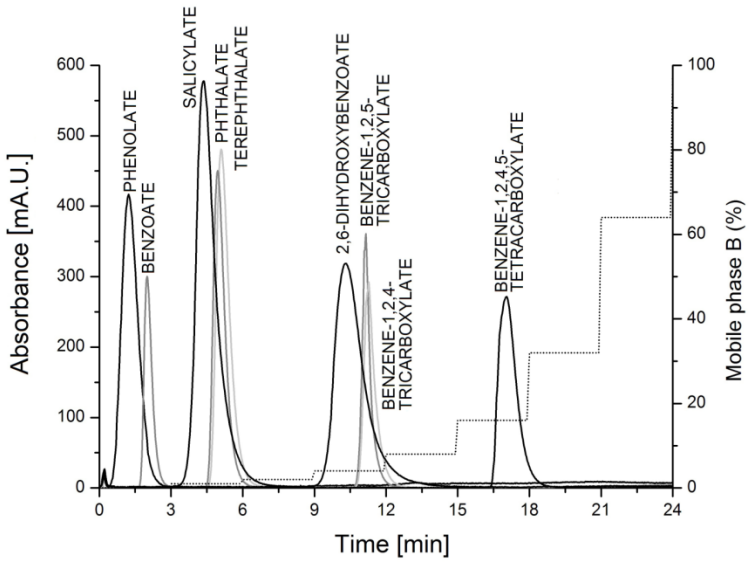

Fig 4. Chromatographic profiles of selected aromatic acids in 3 minutes steps. For chromatographic conditions see Table 2. Scheme and shape of used stepwise gradient is illustrated on the figure.

As is evident from the Figure 3 the peak shapes of almost all aromatic acids are distorted shoving shoulders. Due to relatively large particles of the used sorbent the described separation system offers with low efficiency, hence some advise effects like peak shouldering are visible as expected. This phenomenon is not occurring in case of profiling of HSs by reason their polydispersity. We supposed that the shouldering is caused by the fact that selected duration of every and each gradient step is too short with respect to individual peak base width, what means that substances are eluted by the steep boundary of the two neighbouring gradient steps.

Therefore prolongation of the gradient step duration from 2 minutes to 3 minutes was suggested and tested. The change has resulted in substantial improvement of the peak shapes as can be seen in the Figure 4. Improved gradient shape resulted also in improvement of such chromatographic figures-of-merit as are retention time stability and peak area reproducibility (see Table 2 and Fig. 5.) with retention time relative standard deviation lower than ±1% excepr phenol (±3%) and peak areas RSD better than ±5%. Estimated LODs fall within the limits 31-66 ng and estimated LOQs fall within the limits 100-220 ng per injection.

From the comparison of information coded in Figure 2 and Figure 4 by application simple analogy we can conclude that under the actual separation conditions non-retained or weakly retained fraction of humic substances represents sobstances bearing effective charge from 0 to 1- eluted from the ion exchanger by 5 mmol L^{-1} EDTA $^{4-}$, whereas retained humic substances probably bear effective charge from 3- to more than 4- with the highest signal attributable to the effective charge higher than 4- (eluted by 327 mmol L^{-1} EDTA $^{4-}$).

Table 2. Statistical evaluation of reproducibility of retention times (t_R) and reproducibility of peak areas (A) in 5 consecutive runs measured within two days. In the table the concentration of $EDTA^{4-}$ (c(EDTA)) which eluted given peak was also shown

Analyte	t_R (min)	t_R RSD (%)	A (mAU.s)	A RSD (%)	c(EDTA) (mmol L^{-1})
Phenol	1.068±0.036	3.34	337.6±9.7	2.87	5
Benzoic acid	1.994±0.011	0.55	122.8±4.9	3.99	5
Salicylic acid	4.338±0.037	0.85	573.3±19.5	3.40	10
Terephthalic acid	4.953±0.015	0.30	270.3±11.9	4.40	10
Phthalic acid	5.097±0.016	0.31	328.2±14.1	4.30	10
2,6-Dihydroxy-benzoic acid	10.25±0.06	0.59	454.9±22.3	4.90	25
Benzene-1,2,4-tricarboxylic acid	11.16±0.03	0.34	148.0±4.9	3.31	25
Benzene-1,2,5-tricarboxylic acid	11.28±0.03	0.27	165.9±5.8	3.50	25
Benzene-1,2,4,5-tetracarboxylic acid	16.95±0.03	0.18	236.5±8.0	3.38	84

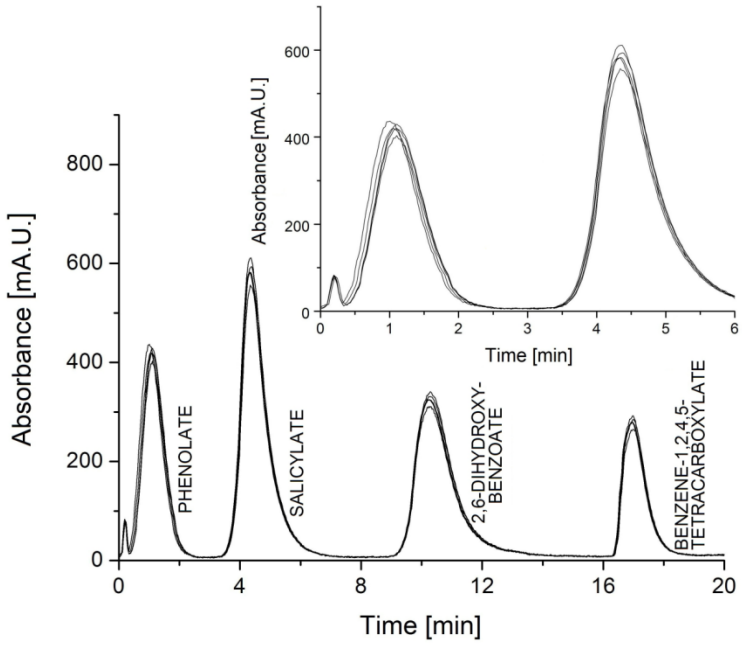

Fig 5. Overall reproducibility of 5 overlayed chromatographic profiles of selected standard of aromatic acids (from 1.00 to 5.00 mg mL^{-1}, individually) measured within two days. For chromatographic conditions see Table 2.

4. Conclusion

The developed anion exchange chromatography allows a relatively simple profiling humic substances, soils alkali extracts and selected aromatic acids. Mobile phase composed of EDTA titrated to pH 12.0 by sodium hydroxide at various concentrations levels with a run time (38 min) and stepwise gradient elution used are advantageous and made the routine humic substances characterization easy. Among the significant advantages of this method are simplicity, sufficient precision ensuring that it is suitable for combinations with other HPLC

separation mechanisms (e.g. SEC, RP-HPLC) for comprehensive characterization or fractionation of various types of humic substances, soil extracts and aromatic acids.

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