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ORIGINAL ARTICLE

Chromatographic Separation of Hydrophilic Organophosphates on a Porous Graphitized Carbon Sorbent Hypercarb Using an Aqueous Solution of Formic Acid As a Mobile Phase

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Abstract—The possibility of the elution of hydrophilic organophosphorus substances using a concentration gradient of formic acid in an aqueous mobile phase on a Hypercarb porous graphitized carbon sorbent is studied. The analytes are detected on a monoquadrupole mass spectrometer. The retention of analytes is studied by varying the composition of the mobile phase before injection of the sample solution. It is shown that the effect of the stepwise gradient elution on the retention of analytes is primarily due to the state of the sorbent surface rather than the elution ability of the phases. The displacement "quasi-ion exchange" mechanism of analyte retention seems to be most probable.

Keywords: liquid chromatography, mass spectrometry, Hypercarb porous graphitized carbon sorbent, alkylphosphonic acids, organophosphorus pesticides

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Hydrophilic organophosphorus compounds are a large group of substances, many of which are found in everyday life. Organophosphorus herbicides and insecticides, such as glyphosate (Gly), glufosinate (Glu), acephate (ACE), and omethoate (Ome), can be carcinogenic, whereas methamidophos (Meth) and monocrotophos (Mon) are toxic and banned in many countries. Hydrophilic organophosphorus compounds also include the transformation products of nerve agents (NAs): alkylphosphonic acids (APA) and *O*-alkylalkylphosphonic acids (*O*-AAPA). Methylphosphonic acid (MPA) and its homologs, ethyl (EPA) and propylphosphonic (PPA) acids, as well as ethyl (EtMPA), isopropyl (iPrMPA), and isobutylmethylphosphonic (iBuMPA) acids act as markers for the use of chemical weapons. It is necessary to develop simple, effective, and reliable methods to detect these pesticides and chemical agents, whose decomposition products are APA and *O*-AAPA, in environmental objects, food products, and biomedical and any other objects because of the danger they pose and their toxicity.

High-performance liquid chromatography (HPLC) operated in the reverse phase (RP), as well as ionic and HILIC modes is frequently utilized as a method to detect these analytes [1–3]. It should be noted that these substances have low absorption in the UV and visible regions. They are also not capable of fluorescence; thus, they can be selectively detected on a mass spectrometer exclusively. Although this method does not require the derivatization of analytes, major problems arise because of their high polarity: these substances are poorly retained in most commercially available phases. Moreover, the determination with MS detection in ion chromatography is only possible after a labor-intensive procedure to decrease the influence of the accompanying ions. Gas chromatography-mass spectrometry (GC-MS) is also often used to detect these analytes [4]. A labor-intensive and time-consuming derivatization step, however, is necessary before the GC analysis because of the low volatility of APA, *O*-AAPA, and some pesticides.

It is known that the Hypercarb sorbent retains polar compounds much better than C18 silica gel in RP HPLC; hence, it was proposed to use Hypercarb as a stationary phase for the HPLC separation/determination of hydrophilic organic compounds in our previous works [5–8]. The method proposed in [5, 7, 8] to increase the retention time of hydrophilic organophosphorus analytes by washing the Hypercarb column with water before injection of a sample was used in this study. The range of substances detected was expanded, and the mechanism of retention of hydrophilic organophosphorus compounds on a Hypercarb graphitized carbon sorbent by washing the column with water was studied in more detail. This technique may be extremely useful and will allow better separation of complex mixtures of hydrophilic analytes.

EXPERIMENTAL

Reagents. The deionized water (18.2 MOhm cm) obtained on a Millipore Simplicity installation (Millipore, United States) and a solution of formic acid in water (50 wt %) of HPLC purity (Sigma-Aldrich, United States) were used to prepare the solutions and eluents.

The starting solutions of MPA, EPA, PPA, EtMPA, iPrMPA, iBuMPA, Gly, Glu, Ace, Meth, Ome, and Mon with a concentration of 1 mg/mL were used. Table 1 shows the structures and properties of the analytes. All substances were produced by Sigma-Aldrich (United States). Working solutions of a mixture of analytes with a concentration of 70 μg/mL were prepared by the serial dilution of the intial solutions. The initial and working solutions were stored in the dark at +4°C.

Equipment. A Shimadzu liquid chromatography-mass spectrometer equipped with the following modules was used: an LCMS-2020 quadrupole mass spectrometer with the electrospray ionization (ESI) of analytes, two LC-20AD HPLC pumps, a DGU-20A degasser, a CBM-20A controller, a SIL-20AC autosampler, and a CTO-20AC thermostat. The following parameters of the MS detector and the Shimadzu LCMS-2020 interface recommended by the equipment manufacturer were used to detect analytes: intensity registration time at m/z (SIM event time) of 0.2 s, detector voltage of 1.55 kV, interface voltage of 4.5 kV, DL voltage of 0 V, DL temperature of +250°C, nebulizing gas flow 1.5 L/min, heat block temperature of +400°C, and drying gas flow of 15 L/min. The analytes were detected in the negative positive ion detection mode. Table 1 shows the parameters of the m/z registration of analyte ions for the mass spectrometer. The chromatographic parameters were calculated with a LabSolutions program. A column with a Hypercarb sorbent (Thermo Scientific, United States) (3.2 × 2.1 mm, 5 μm) was used for HPLC separation. The pH value was measured with an Ekoniks Ekspert pH meter.

Chromatographic detection conditions. A 0.1% aqueous solution of formic acid was used as an eluent (phase B), whereas deionized water, water saturated with CO_2, and a 0.5 mM aqueous solution of NH_3 saturated with CO_2 were used as washing solutions (phase A). The eluent flow rate was 0.2 mL/min, and the column temperature was 30°C.

RESULTS AND DISCUSSION

A technique proposed previously for the preliminary equilibration of a column with water with the subsequent stepwise concentration gradient of formic acid during the separation of analytes on a porous graphitized carbon sorbent makes it possible to improve the shape of analyte peaks: a decrease in the width of peaks and an increase in their height. Two elution modes were selected on the basis of previous results [8], where the step gradient showed better separation of carboxylic acids compared to the isocratic mode and linear gradient: the isocratic mode (for comparison) and the step gradient mode. The elution phase was 100% phase B in the isocratic mode. However, the step gradient consisted of two sections: 100% of phase A was passed before the injection of analytes, and 100% of phase B after injection, where the time point with sample injection and the simultaneous change of the mobile phase was taken as the 0th min. Table 2 shows the conditions for isocratic and stepwise gradient chromatographic separations with column washing before injection (t_w) for 5 min as an example. It was found that the duration of washing the column with phase A before injection plays an important role in the case of the step gradient. In our opinion, when the column is washed before the injection of analytes with clean water (phase A) for less than 5 min, a certain amount of formate ions remains on the sorbent surface, and the retention of analytes is similar to the isocratic mode, in which no washing was performed at all. As a result, the minimum time required for column equilibration before sample injection was 5 min (Fig. 1). The further increase in duration of this stage had an insignificant effect on the retention time of analytes.

It was impossible to achieve complete separation of the following pairs of substances for a model mixture of analytes during the elution in the isocratic mode with 0.1% formic acid: MPA/EPA and Ace/Gly/Glu. Moreover, it was found that the step gradient of formic acid during washing with clean water before injection had an effect on the retention of phosphonic acids, glyphosate, and glufosinate to the same extent. As a result, the retention time of the analytes increased with the improvement of the separation of the MPA/EPA pair compared to the isocratic mode (Table 3). In addition, it was shown for organophosphorus pesticides that the gradient elution with formic acid significantly influences the retention pattern of acidic analytes, which are found as anions in a neutral environment. This can be explained by the weak anion-exchange properties of the Hypercarb sorbent. However, phosphorus-containing hydrophilic analytes (acephate, methamidophos, omethoate, and monocrotophos) have no hydroxyl groups (Table 1), and they are found as cations under our experimental conditions due to the amino groups in the molecules. The formic acid gradient had an insignificant effect on their retention time, and the retention time for these pesticides did not depend on either the duration of washing or the composition of the washing phase A. This may indicate that these cations are not retained on the surface of the sorbent due to ionic forces, and their separation proceeds according to the RP mechanism. At the same time, the chromatographic peaks of methamidophos and monocrotophos had an extremely low intensity relative to the other analytes, and a significant distortion of symmetry was observed both in the isocratic mode and in the step gradient, and therefore they are not shown in Fig. 2.

Table 1. Structures of analytes, their lipophilicity parameters (log *P*), and *m*/*z* values selected for mass spectrometric registration of analyte ions

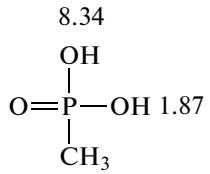
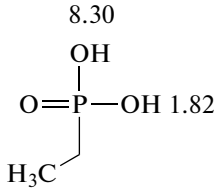
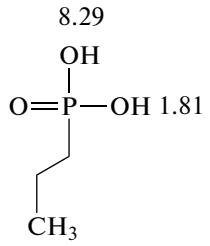
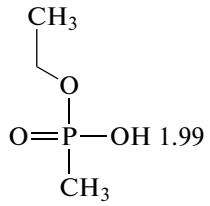
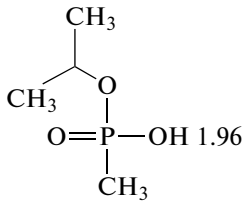
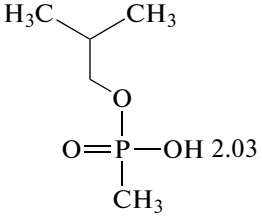
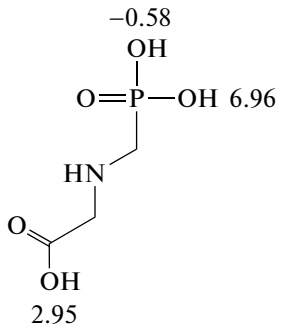
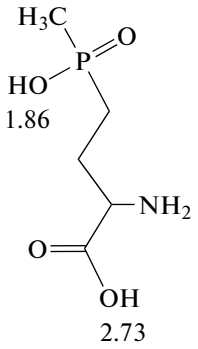
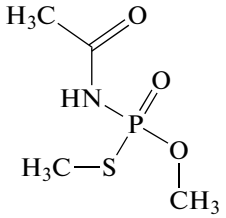
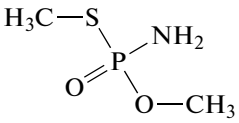
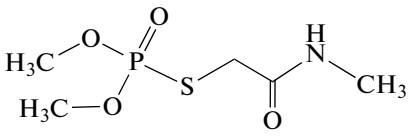
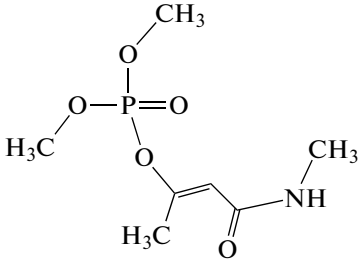
Analyte	Structure with pK_a of functional groups	*m*/*z*	log *P*
MPA		−95	−1.6
EPA		−109	−1.2
PPA		−123	−0.7
EtMPA		−123	−0.8
iPrMPA		−137	−0.3
iBuMPA		−151	0.1

Table 1. (Contd.)

Analyte	Structure with pK_a of functional groups	m/z	log P
Gly		−168	−4.6
Glu		−180	−5.0
Ace		+184	−0.8
Meth		+143	−0.9
Ome		+214	−0.9
Mon		+224	−0.2

It is assumed that the retention of acidic analytes on the Hypercarb sorbent under these conditions is probably due to the hypothetical displacement "quasi-ion exchange" mechanism of the analyte elution and the influence of the composition of the mobile phase on the state of the sorbent surface. In our opinion,

Table 2. Conditions for isocratic and step gradient chromatographic separations with column washing before injection

Stage	Column washing, from 5th to 0th min	Sample injection, 0th min	Analysis termination, 25th min
Isocratic mode	100% phase B	100% phase B	100% phase B
Step gradient	100% phase A	100% phase B	100% phase B

Table 3. Resolution of some carboxylic acid pairs depending on selected elution mode

Pair of analytes	Resolution (Rs)			
	composition of washing solution			
	without washing	H_2O*	saturated CO_2*	saturated CO_2, NH_3 * aquatic
MPA/EPA	0.82	1.09	0.54	0.43
PPA/EtMPA	1.32	1.24	0.84	1.00
Gly/Glu	0.50	0.34	0.33	0.32

* Washing duration was 6 min.

when formic acid solutions are used as the mobile phase, the formate ions partially remain on the surface of the sorbent due to its weak anion-exchange properties. Formate ions are washed out from the surface of Hypercarb during the washing process with water, and the elution ability of pure water is significantly worse. It increases the retention of acidic analytes. Some anions (bicarbonate and hydroxide ions) capable of replacing formate ions on the surface of the sorbent were therefore selected to study the influence of the composition of the washing mobile phase on the retention time of hydrophilic organophosphorus substances. Hydrocarbonate ions were obtained by dissolving carbon dioxide in water. The experiments included the following mobile phases to wash a column: H_2O (pH 6.2), H_2O saturated with CO_2 (pH 3.9), and H_2O saturated with CO_2 with the subsequent addition of an NH_3 aqueous solution (pH 8.3). It should be noted that in the stepwise gradient mode, all analytes with acidic groups are initially in the ionic form due to the fact that the pH of phase A is similar to that of a neutral one. When the concentration of formic acid increases and the pH of the mobile phase decreases to 2.7, the analytes transform into the molecular form, whereas glyphosate and glufosinate transform into the zwitterionic form. When the step gradient is used, the mechanism to retain analytes during the initial separation stage is therefore mainly ionic. When the analytes transform into the molecular form, the transition proceeds according to the RP mechanism. As a result, the step gradient influences the retention of analytes in various degrees to improve the resolution of the substances studied. In addition, when the step gradient was used, the width of most peaks

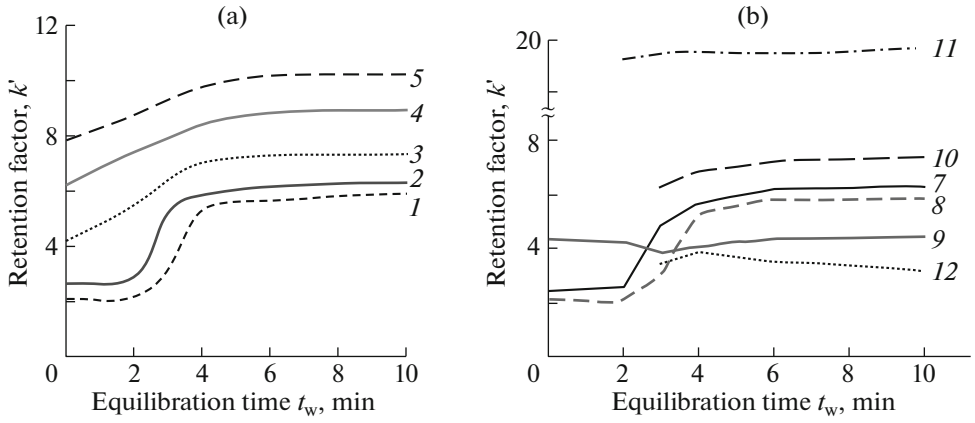


Fig. 1. Dependence of analyte retention factor (k') on duration of column equilibration (t_w) with water. (a) Acids separated: (*1*) MPA, (*2*) EPA, (*3*) PPA, (*4*) EtMPA, and (*5*) iPrMPA. (b) Pesticides separated: (*7*) Gly, (*8*) Glu, (*9*) Ace, (*10*) Meth, (*11*) Ome, and (*12*) Mon. S_{analyt} = 70 μg/mL; 3 μL of solution was injected.

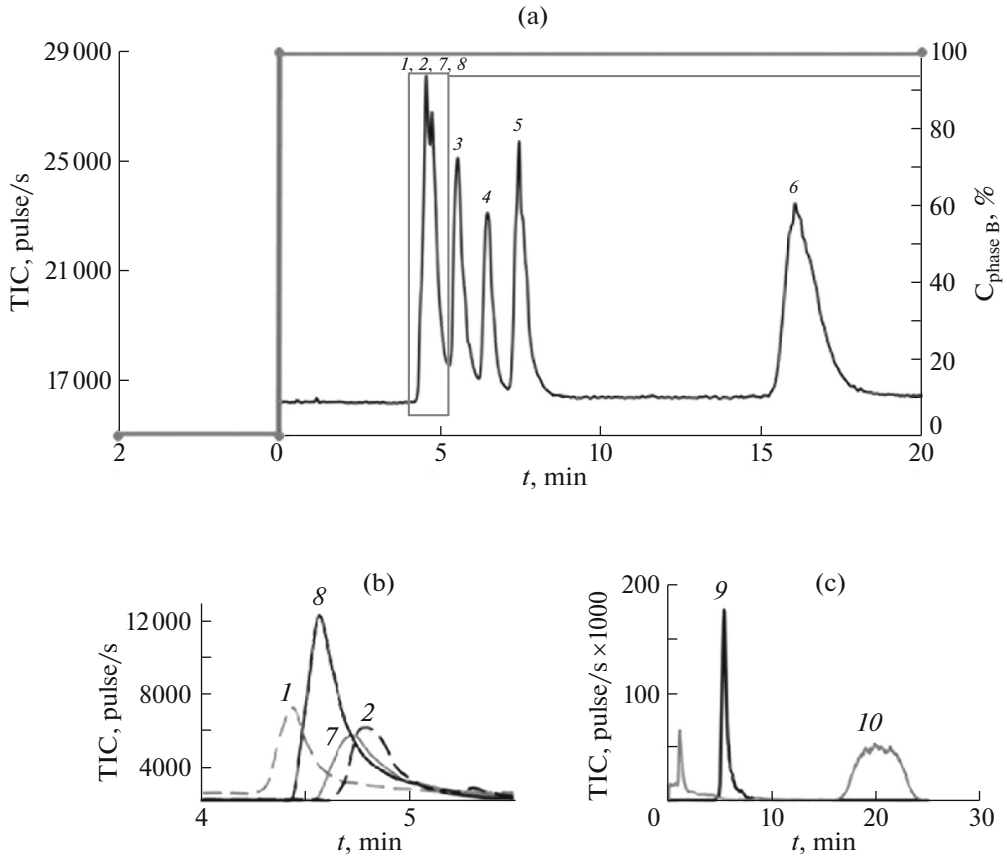


Fig. 2. Chromatogram obtained by separating carboxylic acids on column with porous graphitized carbon. Acids separated: (*1*) MPA, (*2*) EPA, (*3*) PPA, (*4*) EtMPA, (*5*) iPrMPA, and (*6*) iBuMPA. Pesticides separated: (*7*) Gly, (*8*) Glu, (*9*) Ace, and (*10*) Ome. (a) Chromatogram for total ion current of analytes (*1*)–(*8*), (b) overlay of chromatograms of analytes (*1*), (*2*), (*7*), and (*8*), (c) overlay of chromatograms of analytes (*9*) and (*10*). $C_{analyte}$ = 70 μg/mL; 3 μL of solution was injected.

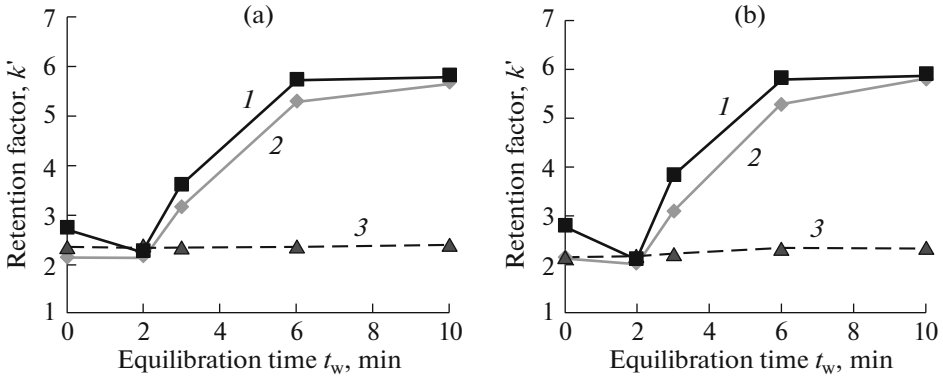


Fig. 3. Dependencies of retention factor of (a) MPA and (b) Glu on duration of equilibration of column with: (*1*) water without additives, (*2*) water saturated with CO_2, and (*3*) water saturated with CO_2 and 5 mM NH_3 aqueous solution.

decreased and their intensity increased, which also influenced the resolution of chromatographic peaks.

Similar changes in the retention time for acidic analytes were observed in the case of the "water without additives" and "water saturated with CO_2" wash phases. Figure 3 shows the typical relationships between the retention factors of these analytes and the time of washing the column before injection. If the column is washed with water saturated with CO_2 (pH 3.9), the vast majority of the additive is in molecular form (H_2CO_3) and weakly interacts with the surface of the sorbent charged positively. The formic acid

that remains from the previous experiment is therefore washed out from the column in these two washing options, and the analytes are more strongly retained on the surface during the subsequent separation of organic acids. The addition of a hydrogen carbonate modifier (HCO_3^-) to the washing mobile phase at pH 8.3 led to the situation where the retention time of the analytes remained unchanged compared to that of the isocratic mode. In the case of washing with a solution containing bicarbonate in an alkaline medium, the formate residues are probably replaced from the sorbent surface by the bicarbonate, and the washing phase has a similar elution force to 0.1% formic acid, due to which the retention times of the analytes remain almost the same.

In summary, it was shown that the sorption of hydrophilic organophosphorus substances with acidic groups probably proceeds according to the displacement quasi-ion exchange mechanism.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This work does not contain any studies involving human and animal subjects.

CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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