JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2011, 59, 803–808 803
DOI:10.1021/jf103169p

Enantiomeric Discrimination and Quantification of the Chiral Organophosphorus Pesticide Fenamiphos in Aqueous Samples by a Novel and Selective ^{31}P Nuclear Magnetic Resonance Spectroscopic Method Using Cyclodextrins as Chiral Selector

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A rapid, selective, and accurate quantitative ^{31}P nuclear magnetic resonance (^{31}P NMR) spectroscopy method was used for the chiral recognition of the racemic organophosphorus pesticide fenamiphos using chiral solvating agents (CSAs). Six neutral cyclodextrins (CDs) (α-CD, β-CD, methyl-β-CD, hydroxyethyl-β-CD, hydroxypropyl-β-CD, and hydroxypropyl-γ-CD) and two anionic CDs (carboxymethyl-β-CD and carboxyethyl-β-CD) were selected for these experiments. The shift displacement values ($\Delta\delta$), after addition of each of the eight CDs in the highest possible molar ratio to a guest, were recorded. The results showed that β-CD and hydroxypropyl-β-CD were the best chiral solvating agents for the enantiomeric discrimination of fenamiphos. Two-dimension rotating frame nuclear Overhauser spectroscopy (ROESY) was used to investigate the structure of the β-CD−fenamiphos inclusion complex in aqueous solution. To determine the fenamiphos enantiomers, a calibration curve was drawn for two enantiomers over the range of 0.05−0.25 mg mL^{-1}. The limits of detection (S/N = 3) were obtained as 0.0068 and 0.0060 mg mL^{-1} for fenamiphos enantiomers. The recovery studies were performed on aqueous real samples ranging from 94 to 107% with coefficients of variation of ≤9%.

KEYWORDS: Enantiomeric discrimination; ^{31}P nuclear magnetic resonance; organophosphorus pesticide; cyclodextrins

INTRODUCTION

Organophosphorus pesticides (OPPs) are toxic to mammals. Existing regulatory limits for human exposure are based on inhibition of acetylcholinesterase (AchE) either in experimental animals or in humans (*1*, *2*). A number of OPPs are chiral, and their enzyme inhibition activity, toxicity, and biodegradability depend on the absolute configuration of their phosphorus, carbon, or sulfur chiral atoms (*3*, *4*). In a few cases, such as fenamiphos [(*O*-ethyl-*O*-(3-methyl-4-methylthiophenyl)isopropylamidophosphate)] (*5*), one enantiomer is much more toxic than the other, so it would be desirable to use a single enantiomer in the commercial formulation. Also, pesticides are released into the environment; transformation products may be chiral themselves (*6*), derived from chiral pollutants, and chiral products can also be transformed from achiral pollutants, which exhibit potential enantioselectivity in environmental behavior and toxicological significance (*7*). Thus, to discriminate chiral pesticides or their transformation products in environmental media and, also, assess the enantiopurity of commercial pesticide formulations, analytical methods must be developed.

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There are numerous methods for enantiomeric analysis of OPPs, such as gas chromatography (GC) (*8*), high-performance liquid chromatography (HPLC) (*9*), and capillary electrophoresis (CE) techniques (*10*, *11*). These analytical methods have been validated for the determination of racemic mixtures of fenamiphos in environmental samples (*12*, *13*), but only a few HPLC methods through the usage of a chiral stationary phase (*14*−*16*) or a CE method (*17*) allows chiral separation of fenamiphos enantiomers.

Among the nonchromatographic techniques enabling enantiomeric analysis, nuclear magnetic resonance (NMR) spectroscopy using milligram levels of analyte and about 500 μL of deuterated solvent has been shown to be a facile and environmentally benign tool (*18*, *19*). Unlike chromatography or CE techniques, NMR is an analytical method that allows the measurement of the enantiomeric ratio without the separation of the enantiomers (*20*) while simultaneously providing direct structural information about the nature, conformation, and dynamics of the diastereomeric complexes of chiral molecules in a way that is not accessible from separation methods. In the case of organophosphorus compounds, NMR also offers an extra possibility for observing ^{31}P nuclei characterized by a wide range of chemical shifts and simplicity of the spectra, which are independent of the C,H-complexity of the molecule (*21*, *22*). The other advantage of ^{31}P

Published on Web 12/30/2010

NMR spectroscopy for enantiopurity determination is the possibility of avoiding the spectral complications caused by the presence of the reagent excess or non-phosphorus admixtures (*23*).

Discrimination of enantiomers using the NMR technique requires an effective chiral additive to combine with the sample to form diastereomeric species, which need to appear different in some of their NMR signals. The chiral additive is generally divided into chiral shift reagent (CSR), chiral derivatizing agent (CDA), and chiral solvating agent (CSA) (*24*). Cyclodextrins (CDs) are small cyclic polysaccharides that form a cone-shaped cavity with six, seven, or eight glucopyranose units for α-, β-, or γ-CD, respectively, and were proposed in early 1977 as CSA in NMR (*25*). Inclusion complexation is the driving interaction in chiral recognition by CDs (*26*). Nuclear Overhauser enhancements (NOE) and, in particular, two-dimensional (2D) NOE experiments in the rotating frame (ROESY), which give positive and enhanced NOEs over the whole molecular range, have been useful for examining the through-space interactions between analyte and CD nuclei in CD−analyte complex (*27*)

In this paper a novel, simple, and selective method for enantiomeric discrimination and quantification of chiral OPPs in aqueous media by means of ^{31}P NMR spectroscopy with the use of cyclodextrins as chiral selector has been described. A few chiral OPPs (methamidophos, acephate, and fenamiphos) were examined, and different chiral selectors were evaluated in the preliminary study to achieve the enantiomeric resolution of these OPPs. The optimization of the method was performed by taking into account the influence of CD type, molar ratio of CD to guest, and temperature in the discrimination of pesticide enantiomers. To understand the interaction of chiral OPPs and CD, 2D ROESY was used. Finally, the selected experimental parameters were optimized for the quantitative NMR method, and fenamiphos enantiomers could be determined in aqueous real samples.

MATERIALS AND METHODS

Chemicals. The (±)-methamidophos, (±)-acephate, and (±)-fenamiphos standards were obtained from Reidel-de Haën (Seelze, Germany). The chiral selector α-cyclodextrin (α-CD) was supplied by Aldrich (Steinheim, Germany). β-Cyclodextrin (β-CD), (2-hydroxyethyl)-β-cyclodextrin (HE-β-CD), carboxymethyl-β-cyclodextrin sodium salt (CM-β-CD), and (2-carboxyethyl)-β-cyclodextrin sodium salt (CE-β-CD) were purchased from Fluka (Steinheim, Germany). Methyl-β-cyclodextrin (Me-β-CD), (2-hydroxypropyl)-β-cyclodextrin (HP-β-CD), and (2-hydroxypropyl)-γ-cyclodextrin (HP-γ-CD) were obtained from Sigma (Steinheim, Germany). Deuterium oxide was bought from Merck (Darmstadt, Germany). Apple−banana juice was provided from a local market, and drinking water sample was collected from Tehran piped water.

^{31}P NMR Spectroscopy. All ^{31}P NMR spectra were recorded on a Bruker DRX 500 AVANCE operating at 202.456 MHz for the phosphorus-31 nucleus with a dedicated 5 mm QNP probe and running Topspin1.3 software using 500 μL of samples. In all experiments, a known amount of D_2O (100 μL) was added as an internal field frequency lock. The chemical shifts (δ) were reported in parts per million (ppm) considering the resonance peak of 85% H_3PO_4. Spectra were collected in 64000 data points over a 100000 Hz spectral width, with a 90° pulse width with 128−256 scans. The longitudinal relaxation times (T_1) of fenamiphos and acephate as internal standard were determined by the inversion−recovery pulse sequence method (*28*), using the T_1 cal Bruker program and were obtained as 1.18 and 4.85 s for fenamiphos and acephate, respectively. The acquisition time of 0.328 s was followed by a relaxation time delay of 12 s to ensure full T_1 relaxation. The probe temperature was 25 °C for quantitative analysis of fenamiphos.

NMR processing for final solutions of all samples consisted of phase correction (performed manually for each replicate) and the baseline correction over the entire spectral range. Areas of peaks were measured by electronic integration of expanded regions around the selected resonances, using an integral limit of ±20 Hz around the corresponding signals. The results were presented as a difference of ^{31}P chemical shift (Δδ) of two diastereomers of CD−analyte complexes in the case of NMR measurements.

The 2D rotating-frame Overhauser enhancement spectroscopy (ROESY) spectrum with a BBI probe operating at 500.13 MHz for proton was obtained during 13.75 h by using the following conditions: sweep width window, 5122.951 Hz; acquisition time, 0.200 s; relaxation delay, 2 s; scan, 80; spin−lock mixing time, 200 ms. Offset compensation was used to eliminate the dependency of the amplitude of ROE effect cross peaks on the transmitter frequency offset. States−Haberkorn phase cycling with 2048 data points in F_2 and 1024 data points in F_1 was used to acquire the data, which were processed using linear prediction in F_1, with Gaussian apodization in both dimensions.

Sample Preparation. In the OPP enantiomeric discrimination studies, the appropriate amount of the OPPs (methamidophos, acephate, and fenamiphos, typically 1.77, 1.36, and 0.82 mM, respectively) was weighed and dissolved in adequate water containing 20% w/w deuterium oxide. These solutions were transferred into NMR tubes, with various amounts of CDs having high molar ratio to OPPs.

In the case of fenamiphos, nine aqueous solutions having molar ratios of 0.60, 0.80, 1.00, 1.25, 2.14, 5.00, 9.00, 11.50, and 16.50 CD/fenamiphos were prepared in the NMR tube (final volume = 500 μL containing 20% w/w deuterium oxide) so that the total concentration of the interacting species in the solution was kept constant to measure the induced chemical shifts of the suitable CDs and fenamiphos signals.

To quantify fenamiphos enantiomers in aqueous samples, standard stock solutions of (±)-fenamiphos and acephate as internal standard (I.S.) were obtained by dissolving 1.6 mg of (±)-fenamiphos and 3.0 mg of acephate in 2.0 mL of deionized water separately to give final concentrations of 0.8 mg mL^{-1} (±)-fenamiphos and 1.5 mg mL^{-1} acephate. The calibration solutions were prepared daily by appropriate dilution of stock solution to obtain 0.1−0.5 mg mL^{-1} solutions of (±)-fenamiphos and 0.15 mg mL^{-1} acephate. The optimal amount of β-CD was added into the NMR tube (500 μL final volumes). All experiments were carried out in triplicate.

Method Validation. The quantification of the fenamiphos enantiomers was performed using the internal standard method with the integration of phosphorus signal of each fenamiphos enantiomer related to the phosphorus signal of internal standard in each calibration solution. Phosphorus peak area ratios were plotted against the corresponding fenamiphos enantiomer concentration, and a linear regression variance analysis was performed to determine linearity and correlation coefficients. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated by using signal-to-noise (S/N) ratios of 3 and 10, respectively. Accuracy was assessed by determining the concentration of each enantiomer of fenamiphos in water samples relative to the known concentration added (0.128, 0.248, and 0.472 mg mL^{-1} of (±)-fenamiphos; n = 3 for each case). Precision was determined by utilizing the coefficient of variation (CV %) of the within-day (n = 3) and between-day (n = 3) variations. Recovery studies were carried out by dissolving of 0.18 mg of (±)-fenamiphos and 0.37 mg of acephate in 400 μL of apple−banana juice and drinking water, and then these solutions were transferred into NMR tubes and mixed with 100 μL of deuterated water. ^{31}P NMR spectra of these samples were carried out in triplicate without any sample preparation.

RESULTS AND DISCUSSION

Effect of CD Type on Enantiomeric Discrimination of OPPs. Enantiomeric recognition of three chiral OPPs (methamidophos, acephate, and fenamiphos) was investigated in a ^{31}P NMR system using different CDs as chiral selectors. These CDs were chosen due to differences in the size of their inner cavities (α-, β-, and γ-CDs) and substitution of their rim (Me, HP, HE, CM, and CE substitutions). The structures of selected OPPs and the cyclodextrins used are shown in **Figure 1**. To evaluate the chiral discrimination abilities of these chiral selectors, ^{31}P NMR spectra in the highest possible molar ratio of each cyclodextrin to each guest were recorded. In the cases of methamidophos and acephate, all studied CDs could not discriminate the enantiomers of these two

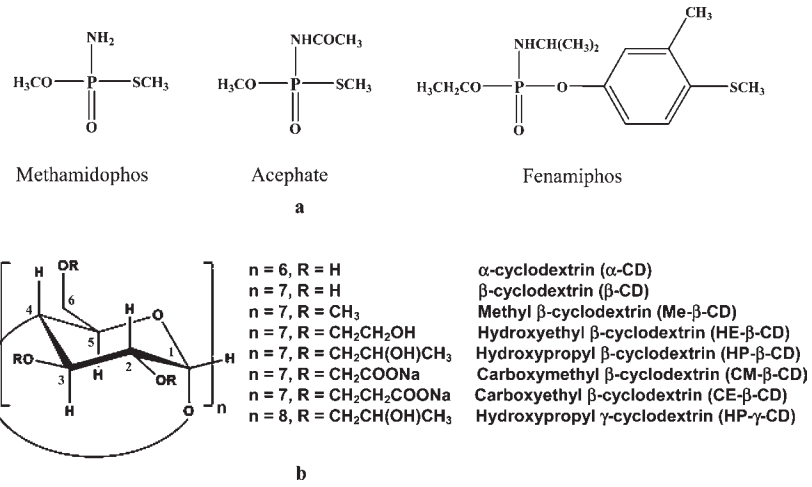

Figure 1. Structures of (**a**) selected chiral organophosphorus pesticides and (**b**) cyclodextrins used as chiral selectors.

Table 1. Chemical Shift Displacement ($\Delta\delta$) of the Fenamiphos in the Presence of Different CDs in the Highest Possible Molar Ratio by ^{31}P NMR Spectroscopy (202 MHz) in Water

CDs	Δδ (ppm)	Spectrum
α-CD	0.00	
β-CD	0.1206	
Me-β-CD	0.0878	
HE-β-CD	0.0915	
HP-β-CD	0.1199	
CM-β-CD	0.0884	
CE-β-CD	0.0993	
HP-γ-CD	0.0531	

compounds, but when dissolved as a racemic mixture of the fenamiphos in the solution containing CDs, baseline separation for two diastereomeric complexes of CD−fenamiphos was observed in the most studied CDs. The results are summarized in **Table 1**. These results can be compared on the basis of two parameters: cavity size and substitution of CDs.

As can be seen, α-CD did not enable the enantiomeric resolution of fenamiphos, and by using HP-γ-CD ($\Delta\delta$ = 0.0531) only partial resolution was obtained. On the other hand, β-CD and different substitutions of it discriminated two enantiomers of

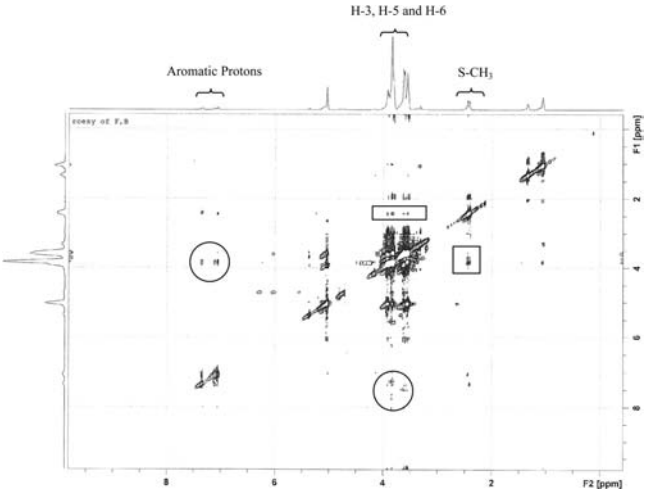

Figure 2. NMR spectrum (500 MHz, 2D ROESY) of (±)-fenamiphos (2.64 mM) after addition of β-CD in a 1:11.5 molar ratio.

fenamiphos. These results proved that α- and γ-CDs annulus are too small and large to accommodate the guest, respectively.

Comparison of the results of different substituents of β-CD on enantiomeric discrimination of fenamiphos showed that the obtained chemical shift variations ($\Delta\delta$ values) are for Me-β-CD < CM-β-CD < HE-β-CD < CE-β-CD < HP-β-CD < β-CD. It seems that decreased hydrogen bond donating of substituent in Me-β-CD, and the presence of charge in CE-β-CD and CM-β-CD on the cyclodextrin annulus, can be effective in enantiomeric recognition. On the basis of these results, β-CD and HP-β-CD seem to be the best chiral selectors among those checked and are approximately equal in the magnitude of chiral discrimination.

These behaviors could arise from the fact that the chiral recognition mechanism of fenamiphos with CDs was mainly due to the formation of an inclusion complex through placement of the phenyl of fenamiphos inside the hydrophobic CD cavity. To confirm this hypothesis, 2D ROESY was used to study the inclusion complex of β-CD−fenamiphos and is shown in **Figure 2**. 2D ROESY spectroscopy is capable of showing relatively spatial relationships among protons in a molecule or in a complex of molecules. This experiment utilizes the dipolar interaction between protons at distances of < 5 Å. In this spectrum correlations between H-3, H-5, and H-6 from β-CD with the aromatic protons of fenamiphos indicate that the substituted phenyl ring of the guest molecule was included in the β-CD cavity. Contacts

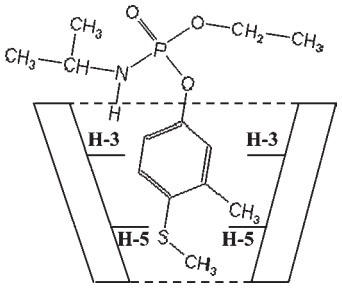

Figure 3. Proposed model for β-CD−fenamiphos inclusion complex according to 2D ROESY results.

Table 2. ^{31}P Shift Displacement Values ($\Delta\delta$) of Fenamiphos Enantiomers in the Presence of β- and HP-β-CDs at Various Ratios

mole ratio CD/fenamiphos	$\Delta\delta$ using β-CD	$\Delta\delta$ using HP-β-CD
0.00	0.000	0.000
0.60	0.021	0.021
0.80	0.024	0.029
1.00	0.029	0.036
1.25	0.044	0.043
2.14	0.051	0.063
5.00	0.078	0.092
9.00	0.089	0.120
11.50	0.104	0.122
16.50	0.101	0.128

between the protons of the $-CH_3$ and $-S-CH_3$ substitutions of the phenyl ring of the guest molecule and inner protons of β-CD also suggest a relatively deep penetration degree. The proposed inclusion complex model is presented in **Figure 3**. These results were also confirmed by the 2D ROESY spectrum of the HP-β-CD−fenamiphos complex.

Effect of Molar Ratio of the Selected CDs to Fenamiphos on Enantiomeric Discrimination. The ^{31}P NMR spectra were measured for the complex of fenamiphos with β-CD and HP-β-CD in different CD/fenamiphos molar ratios (0.60, 0.80, 1.00, 1.25, 2.14, 5.00, 9.00, 11.50, and 16.50). Analysis of the chemical shift variations ($\Delta\delta$ values) for two complexes of fenamiphos with both CD selectors led to a similar pattern (**Table 2**). The results presented in **Figure 2** clearly show that when the CD to fenamiphos molar ratio exceeds 0.6:1, partial resolution of two signals is observed. Maximum values of $\Delta\delta$ were obtained at 11.5 and 16.5 molar ratios of β-CD and HP-β-CD to fenamiphos, respectively. These results show that under equilibrium on the NMR time scale, the free form of fenamiphos is in a dynamic exchange with its complexed forms. For further experiments we have chosen a 11.5:1 β-CD/fenamiphos molar ratio, optimal in consideration of the limited solubility of selected cyclodextrins and limited sensitivity of the NMR spectrometer.

Effect of Temperature on Enantiomeric Discrimination. The effect of temperature on the discrimination of fenamiphos enantiomers was studied within the range of 278−320 K using a solution containing 1.15 mM (±)-fenamiphos and 13.21 mM β-CD. The variation of fenamiphos enantiomer $\Delta\delta$ values in the presence of β-CD as a result of temperature changes is shown in **Figure 4**. It was found that the resolution of isomers was decreased by increasing temperature because the formation of a diastereoisomeric complex probably is an exothermic reaction (*29*). Additionally, there will be a preferential population of specific lower energy conformations at lower temperatures, which means the distinct chemical shifts of the complex are also larger. Lowering the temperature is a useful method for cases in which $\Delta\delta$ may be very small at room temperature, but on the basis

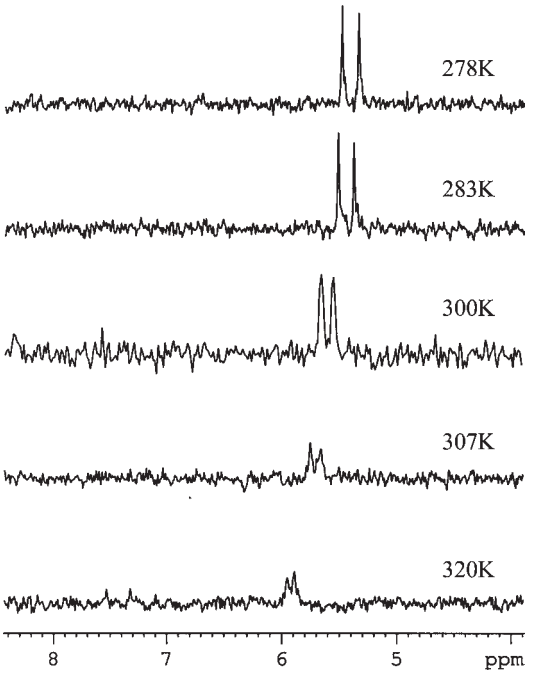

Figure 4. ^{31}P NMR spectra of fenamiphos (1.15 mM) at constant concentration of β-CD (13.21 mM) at different temperatures.

of the obtained results, CSA, β-CD, and HP-β-CD can distinguish between fenamiphos enantiomers at room temperature and are the advised experimental conditions.

Optimization of the Acquisition Conditions for Quantification of the Fenamiphos Enantiomers. To obtain a high accuracy in quantitative analysis, the elapsed time between the successive acquisitions of the spectra must be about 3−5-fold the maximum T_1 value to return all magnetizations to equilibrium between pulses (*30*). These values were measured using the inversion−recovery pulse sequence for frequency offset centered on the signals of fenamiphos and internal standard. On the basis of our results, 12 s was selected as delay time for all ^{31}P NMR analyses. Besides, other factors that influence absolute intensities, such as variable sample volumes, spectrometer performance, and B_1 inhomogeneity over the sample, will cause relative quantification. It is usually for these reasons that quantification in NMR spectroscopy is performed with the aid of an internal standard (*31*). Selection of the internal standard was dictated by high purity, chemical inertness, easy solubility, and good resolution from the analyte signal. In this work, acephate was selected as a suitable internal standard, possessing a sharp singlet ^{31}P signal (located at 33.05 ppm) well resolved from fenamiphos enantiomer signals in the presence of β-CD (located at 5.55 and 5.65 ppm).

Analytical Performance Characteristics. The linearity of the proposed ^{31}P NMR method was tested using standard solutions by increasing concentrations of fenamiphos, in the range of 0.05−0.25 mg mL^{-1} for each enantiomer in the presence of β-CD at its optimum amount. The characterization of calibration curves for fenamiphos enantiomers is shown in **Table 3**. These curves for both enantiomers showed a good linear behavior in the concentration range studied, with correlation coefficients (R^2) of 0.9901 and 0.9696. From these results and by assuming a minimum usable sample volume of 500 μL and 128 scans, the limits of detection (LOD, S/N = 3) under the optimized experimental conditions were approximately 0.0068 and 0.0060 mg mL^{-1} for the two enantiomers of fenamiphos.

The results obtained for fenamiphos enantiomers were accurate and precise as summarized in **Table 4**. The accuracy obtained

Table 3. Linearity and Detection Limit for Analysis of Fenamiphos Enantiomers Using the ^{31}P NMR Method and β-CD as Suitable Chiral Selector

fenamiphos enantiomer	linear range (mg mL^{-1})	regression eq[a]	correl coeff (n = 5)	LOD[b] (mg mL^{-1})	LOQ[c] (mg mL^{-1})
1	0.050−0.250	$I = 225.15C + 3.87$	0.9901	0.0068	0.023
2	0.050−0.250	$I = 256.83C - 1.35$	0.9696	0.0060	0.020

[a] I = relative integral (au); C = concentration of analytes (mg mL^{-1}). [b] LOD is limit of detection at S/N = 3 at 128 scans. [c] LOQ is limit of quantification at S/N = 10.

Table 4. Accuracy and Precision of the ^{31}P NMR Method for the Analysis of Fenamiphos Enantiomers in a Water Sample

		accuracy (recovery, %)		precision (CV[a], %)	
fenamiphos enantiomer	amount added (mg mL^{-1})	within-day (n = 3)	between-day (n = 3)	within-day (n = 3)	between-day (n = 3)
1	0.064	97	96	2	12
	0.124	111	109	10	3
	0.236	100	102	6	4
2	0.064	108	106	6	12
	0.124	107	100	8	5
	0.236	94	88	5	6

[a] Coefficient of variation.

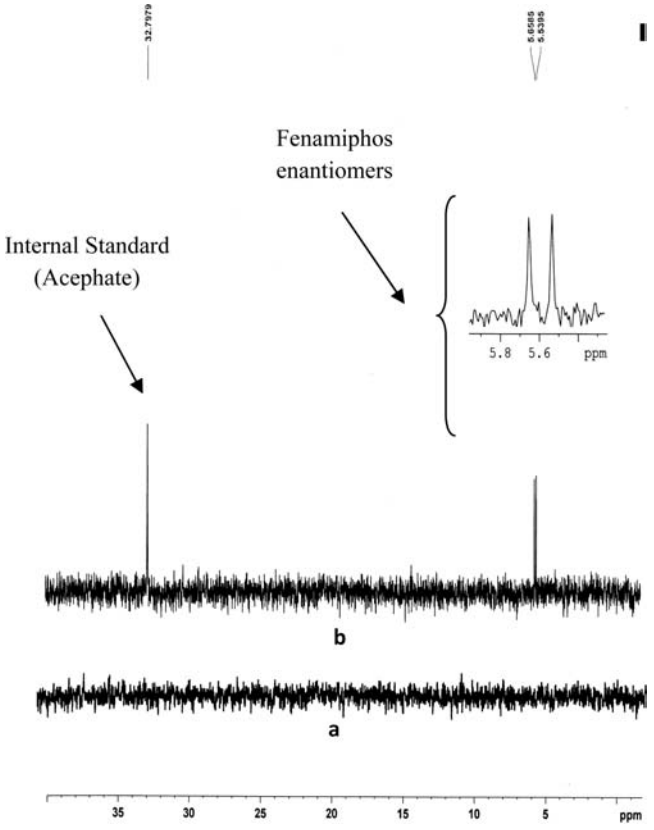

Figure 5. ^{31}P NMR spectrum of (**a**) blank apple−banana juice and (**b**) spiked apple−banana juice with (±)-fenamiphos, acephate, and β-CD at 0.18, 0.37, and 15 mg mL^{-1} concentration levels, respectively.

by means of the concentration of fenamiphos enantiomers measured in the three samples relative to the corresponding known concentrations of fenamiphos enantiomers (0.064, 0.124, and 0.236 mg mL^{-1}; n = 3 for each case) was 95−108%. The precision of the proposed method, as determined by the coefficient of variation of within-day (n = 3) and between-day (n = 3) for the above-mentioned samples, was consistently within 2−10 and 3−12%, respectively. The greatest variance found in quantitative NMR methods arises from sample preparation, geometrical parameters, and integration process. Stringent control of these parameters as well as apodization by the window function, zero filling, and phase, baseline, and drift correction helps to achieve an accurate quantification.

Table 5. Accuracy and Precision of the Fenamiphos Enantiomer Assays in Real Samples in the Presence of β-CD as Suitable Chiral Selector

real sample	fenamiphos enantiomer	amount added (mg mL^{-1})	recovery (%) (n = 3)	CV[a] (%) (n = 3)	t value[b]
apple−banana juice	1	0.09	98	4	0.88
	2	0.09	106	7	1.40
drinking water	1	0.09	107	9	1.26
	2	0.09	94	3	3.68

[a] Coefficient of variation. [b] t value at the 95% confidence level and 2 degrees of freedoms is 4.30.

Analysis of Real Samples. Apple−banana juice and drinking water were selected as models of food and aqueous samples for the determination of fenamiphos enantiomers in them. Recoveries of fenamiphos enantiomers were carried out using an untreated apple−banana juice and drinking water samples fortified at an 0.18 mg mL^{-1} concentration level of (±)-fenamiphos. ^{31}P NMR spectra of blank and spiked apple−banana juice samples are shown in **Figure 5**. The peaks at 5.54 and 5.66 ppm indicate the presence of fenamiphos enantiomers in this real sample without any interference. The recoveries of these analytes from real samples is shown in **Table 5** and lay between 94 and 107% in the two cases; the obtained coefficient of variation values were $<9\%$. Applying Student's t tests on analytical results for two enantiomers of fenamiphos in real samples indicated insignificant differences between measured and real contents at a 95% confidence level (**Table 5**).

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Received for review August 25, 2010. Revised manuscript received December 10, 2010. Accepted December 10, 2010.