

High-performance liquid chromatographic–nuclear magnetic resonance investigation of the isomerization of alachlor–ethanesulfonic acid

Laurie A. Cardoza, Benjamin J. Cutak¹, Jacob Ketter², Cynthia K. Larive*

Department of Chemistry, University of Kansas, 1251 Wescoe Hall Road, Lawrence, KS 66045, USA

Received 1 July 2003; received in revised form 3 September 2003; accepted 19 September 2003

Abstract

The metabolism of the acetanilide herbicide alachlor in soils leads to the formation of alachlor–ethanesulfonic acid (alachlor–ESA) as one of the major transformation products of this compound. The unique structure of alachlor and its metabolites allows the formation of two diastereomers (*s-trans* and *s-cis*) due to the hindered rotation of the amide bond connected to a rigid aromatic ring. Although these stereoisomers do interconvert by rotation about the amide bond, the rate of interconversion is slow allowing separation of the isomers on the chromatographic time scale. Once separated, the unique nuclear magnetic resonance signals of each isomer can be used to monitor the rate of isomerization. This paper reports the on-line separation and detection of the rotational diastereomers using high-performance liquid chromatography–nuclear magnetic resonance (HPLC–NMR) to efficiently measure the isomerization rate of alachlor–ESA.

© 2003 Elsevier B.V. All rights reserved.

Keywords: Nuclear magnetic resonance spectrometry; Kinetic studies; Positional isomers; Alachlor–ethanesulfonic acid; Alachlor; Pesticides

1. Introduction

The acetanilide herbicide, alachlor, has been widely used in the United States for the reduction of broadleaf weeds and annual grasses associated with the production of agricultural crops including corn, soybeans and sorghum. Due to the large-scale use of alachlor and other herbicides in this class there has been significant interest in the processes by which they are transformed and in the identity of their transformation products. The metabolism of alachlor in the soil leads to the rapid production of the polar metabolite alachlor–ethanesulfonic acid (alachlor–ESA) (Fig. 1) as one of its primary transformation products [1,2]. Alachlor and alachlor–ESA have been identified in both ground and surface waters in the Mid-Western region of the USA as a consequence of its agricultural usage [1,3].

The unique structure of alachlor, alachlor–ESA (Fig. 1) and other acetanilide herbicides allows these compounds to

undergo *cis–trans* isomerization (Fig. 2) as a result of hindered rotation of the amide bond with respect to the rigidly substituted aromatic ring. The orientation of the carbonyl moiety and aromatic ring in a relative *cis* and *trans* configuration gives rise to the *s-cis* and *s-trans* nomenclature [4–8]. Although rotational isomers are not as common as other stereoisomers, *Cis–trans* isomerization has been documented in biological [9–12] and synthetic chemical systems, such as the acetanilide herbicides [4–8]. *Cis* and *trans* isomers are often observed in proteins and peptides containing proline and play an important role in protein structure and peptide activity [9–12]. A number of analytical techniques have been used to probe the *cis–trans* isomerization of proline containing peptides including high-pressure liquid chromatography [13,14], capillary electrophoresis [13] and nuclear magnetic resonance (NMR) [12,13].

The complicated proton nuclear magnetic resonance (¹H NMR) spectra of the ESA metabolites of many acetanilide herbicides reflect the different local environments of the protons of each isomer. For example, in the ¹H spectrum of alachlor–ESA, many duplicate resonances are observed corresponding to the protons of the *s-cis* and *s-trans* isomers (Fig. 2) [2]. Although rotational isomers of the acetanilide herbicides and their ESA metabolites can be separated on the chromatographic and electrophoretic time scales, most

* Corresponding author. Tel.: +1-785-864-4269; fax: +1-785-864-5396.

E-mail address: clarive@ku.edu (C.K. Larive).

¹ Present address: Life Science and Technology Center, Sigma-Aldrich, 2909 Laclede Avenue, Saint Louis, MO 63103-3167, USA.

² Present address: Department of Chemistry, University of North Carolina, B5 Venable Hall CB 3290, Chapel Hill, NC 27599-3290, USA.

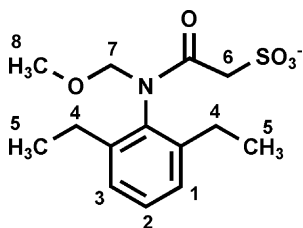


Fig. 1. Structure of alachlor-ESA in its deprotonated form.

analytical methods devised for these compounds have focused on obtaining separations in which the isomers are eluted as a single unresolved peak. Therefore the methods developed generally rely on separations conducted at elevated temperature to increase the rate of interconversion of the isomers [15]. However, because the goal of this study was to measure the rate of isomerization, a new reverse phase separation method was developed to resolve the *s-cis* and *s-trans* isomers at ambient temperatures.

In 1999, Aga et al. used capillary zone electrophoresis (CZE) and ^1H NMR for the analysis of the rotational isomers of acetanilide herbicides and their metabolites [16]. CZE was utilized to evaluate the elution order and relative ratios of the various isomers, while solutions of acetanilide herbicides were investigated by ^1H NMR to obtain the equilibrium constants and determine the first order rate constants for the interconversion of the rotational isomers [16]. A simpler and more direct approach is to use directly coupled high-performance liquid chromatography–nuclear magnetic resonance (HPLC–NMR) for on-line detection of the separated isomers.

HPLC–NMR has been primarily employed for the structure elucidation or identification of eluting analytes, but this technique has also found a niche as a means of investigating reaction rates. Nicholson et al. have demonstrated the utility of this technique to characterize the anomers and positional isomers of a number of fluorobenzoic acid glucuronides [17,18]. Additionally, HPLC–NMR was used to evaluate the rate constants associated with the internal acyl migration of β -1-*O*-acyl 2-fluorobenzoyl glucuronide and *S*-naproxen β -1-*O*-acyl glucuronide [19,20].

This work illustrates the application of stop-flow HPLC–NMR to separate the *s-trans* and *s-cis* isomers of alachlor-ESA and determine the first order rate constants for their interconversion. The direct on-line coupling of HPLC with NMR detection allows the measurement of the

^1H NMR spectra of each separated isomer, facilitating resonance assignment. Furthermore, by trapping the purified isomers in the NMR flow cell, the time dependence of the NMR spectra can be used to follow the return to equilibrium allowing the determination of the rate constants for the interconversion of the *s-trans* and *s-cis* isomers.

2. Experimental

2.1. Chemicals

Alachlor-ESA was synthesized using a previously reported method [2,21]. Sodium formate (NaHCO_2), ammonium formate (NH_4HCO_2), methylammonium chloride (MeNH_3Cl), dimethylammonium chloride ($\text{Me}_2\text{NH}_2\text{Cl}$), and tetramethylammonium bromide (Me_4NBr), were all purchased from the Sigma (St. Louis, MO, USA). Formic acid was purchased from Fisher Scientific (Springfield, NJ, USA). Deuterium oxide ($^2\text{H}_2\text{O}$) with a 99 at.% ^2H and [$^2\text{H}_3$] acetonitrile ($\text{C}^2\text{H}_3\text{CN}$) with a 96–97 at.% ^2H were purchased from Cambridge Isotope Labs. (Andover, MA, USA). The Omnisolv acetonitrile (CH_3CN) was purchased from EM Science (Gibbstown, NJ, USA). Deuterium chloride (^2HCl) prepared as a 20% (w/w) solution in $^2\text{H}_2\text{O}$ with a 99.5 at.% ^2H was purchased from Aldrich (Milwaukee, WI, USA).

2.2. High-performance liquid chromatography

The reversed-phase HPLC separations were performed using Varian ProStar 230 solvent delivery system with a ProStar 330 photodiode array detection (DAD) system (Varian Instruments, Walnut Creek, CA, USA). The separations were monitored at 210 nm and DAD spectra were measured over a spectral range of 200–400 nm. The HPLC chromatograms were processed using the Star Chromatography Workstation software (Varian Instruments). The separations were conducted at ambient temperature (25°C) with a flow rate of 0.5 ml/min using a 150 mm \times 4.6 mm Phenomenex Luna $\text{C}_{18}(2)$ column (Torrance, CA, USA) packed with 3 μm particles. The separations of the alachlor-ESA isomers were performed using 50 μl injection of a 1 g/l alachlor-ESA solution prepared in $^2\text{H}_2\text{O}$. To determine the mobile phase buffer composition that produced the best resolution of the two isomers, a series of aqueous 25 mM formate buffers were evaluated containing sodium, ammonium,

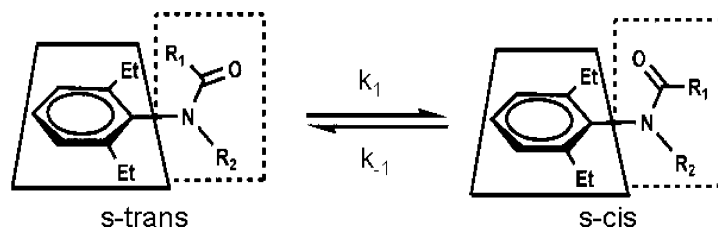


Fig. 2. Isomerization of the amide bond of alachlor-ESA, where $\text{R}_1 = \text{CH}_2\text{-SO}_3^-$ and $\text{R}_2 = \text{CH}_2\text{OCH}_3$.

methyl ammonium, dimethylammonium, or tetramethylammonium as the cation. The buffers were prepared in protonated water and the pH was adjusted to 3.3 with HCl. The effect of the cations on the resolution of the alachlor–ESA isomers was evaluated using an isocratic separation scheme with acetonitrile (30%) and formate buffer (70%). Resolution of the isomers was calculated from the difference in retention times (Δt_r) of the isomer peaks divided by the average of the two base peak widths (avg. w_b). The HPLC–NMR separations were performed using a 25 mM ammonium formate buffer (70%) at p²H 3.7 (pH meter reading of 3.3) [22] and CH₃CN (30%) following the same isocratic conditions described above. All separations described were monitored using a photodiode array detector (Varian Instruments).

2.3. Nuclear magnetic resonance spectroscopy

The NMR experiments were performed at 20.0 °C with a Varian Inova 600 MHz spectrometer. The isomers were conclusively identified from the results of a ¹H–¹H nuclear Overhauser effect spectroscopy (NOESY) experiment with a 1 s relaxation delay, a 0.25 s mixing time and an acquisition time of 0.168 s. The two-dimensional NOESY spectrum was measured for an equilibrium mixture of a 5 mM solution of alachlor–ESA at equilibrium in a 5 mm NMR tube. Solvent suppression was not required in the NOESY experiment because the sample was dissolved in a solution comprised of 30% C²H₃CN and a ²H₂O solution of 25 mM ammonium formate with a p²H of 3.7.

The HPLC–NMR stop-flow experiments used a triple resonance flow probe (Varian NMR Systems, Palo Alto, CA, USA) with a 120 μ l flow cell containing a 60 μ l active volume. A series of ¹H NMR spectra of the separated isomers were collected as an array to monitor the isomerization. The ¹H NMR spectra of each isomer were collected as an arrayed experiment using a pre-acquisition delay (pad) to incorporate a selected time delay between spectral acquisitions. The values of the pad delays were selected so that spectra early in the kinetics experiment were separated by a delay of only 300 s. As the reaction proceeded, the delay between spectra was increased to 600, 1200 and finally 1800 s during the time leading to the equilibrium composition. The time-arrayed spectra were collected using the following parameters; the free induction decays (FIDs) were sampled at 32 000 data points over a spectral width of 9000.9 Hz. An acquisition time of 1.0 s was used. No additional relaxation delay was employed. Each spectrum acquired in the array was composed of 64 transients. The time assigned to the first FID of the series was adjusted to the center of the acquisition window (32 s). The times assigned to the remaining FIDs were calculated by adding 64 s to the pre-acquisition delay between the adjacent FIDs.

Water eliminated through transverse gradients (WET) solvent suppression with ¹³C decoupling was applied to suppress the CH₃CN and HO²H resonances using a Varian pulse program (lc1d). The formic acid from the buffer sys-

tem was used as an internal NMR reference at 8.22 ppm. The FIDs were multiplied by an exponential function equivalent to 1 Hz line broadening and were zero-filled to 32,768 points prior to Fourier transformation. Integrals were measured for the ratios of the *s-cis* and *s-trans* isomers and the ratios of the integrals were plotted versus time using the nonlinear least-squares fitting program Scientist version 2.01 (MicroMath Scientific Software, Salt Lake City, UT, USA). The kinetics data were analyzed using a first order reversible reaction model to obtain the rate constants for the *trans*–*cis* (k_1) and *cis*–*trans* (k_{-1}) reactions (Fig. 2).

3. Results and discussion

3.1. Chromatographic separation of rotational isomers

The slow isomerization of alachlor–ESA due to the hindered rotation about the amide bond allows for the formation of two rotational isomers, which can be separated and differentiated by HPLC–NMR. These rotational isomers are in effect diastereomers on the chromatographic and NMR time scales. However, the determination of rate constants using this method will only be effective if the alachlor–ESA isomers are sufficiently resolved in the chromatographic separation. The separation of the alachlor–ESA rotational isomers was initially optimized with protonated solvents and UV detection. Several different formate buffer cations were tested to identify a buffer system that provided improved chromatographic resolution of the isomers. The initial separation was conducted with a sodium formate buffer (Fig. 3A), which did not generate the necessary resolution. However, the resolution doubled (Table 1) with the use of a variety of ammonium

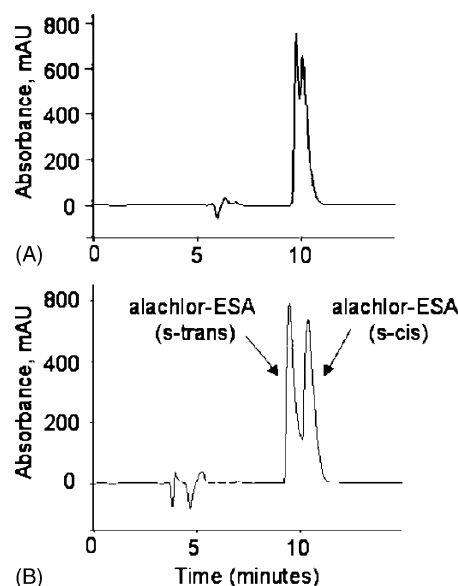


Fig. 3. Separation of alachlor–ESA isomers using a sodium formate (A) and an ammonium formate (B) buffer. The chromatograms were monitored at 210 nm.

Table 1
Chromatographic resolution obtained for the alachlor–ESA isomers using formate buffers containing different cations

Formate buffer cation	Chromatographic resolution
Na ⁺	0.7
NH ₄ ⁺	1.2
MeNH ₃ ⁺	1.3
Me ₂ NH ₂ ⁺	1.3
Me ₄ N ⁺	1.3

cations (NH₄⁺, MeNH₃⁺, Me₂NH₂⁺, Me₄N⁺) probably through ion pairing interactions with the alachlor–ESA. Although the buffers containing methyl-substituted ammonium cations provided slightly better resolution than NH₄⁺, ammonium formate was used for the separation (Fig. 3B) as the methyl protons of the other cations would give rise to additional resonances in the ¹H NMR spectrum that could interfere with observation of the peaks of interest. In addition, the formate proton of this buffer provides a convenient NMR chemical shift reference. The excellent chromatographic resolution obtained with the ammonium formate buffer allowed for the isolation of essentially pure isomers necessary for the NMR analysis.

3.2. NMR spectral analysis and evaluation of dynamic isomerization

The isomers of alachlor–ESA were previously differentiated in a paper by Aga et al. [16]. These authors attributed the one-dimensional NOE interaction observed between protons 4 and 6 to the *s-trans* isomer and those between 6 and 7 to the *s-cis* isomer using the numbering scheme shown in Fig. 1 [16]. We used the two-dimensional NOESY ex-

periment to confirm the identity of the isomers in our solvent system. Although we were unable to observe an NOE cross peak that would conclusively identify the *s-cis* isomer, strong cross peaks between protons 4 and 6 of the *s-trans* isomer were detected allowing the assignment of the *s-cis* isomer by default. The *s-trans* isomer was the first isomer to elute from the column followed closely by the *s-cis* isomer under the conditions of our separation (Fig. 3).

The ¹H NMR spectrum of the equilibrium mixture, shown in Fig. 4, is more complex than expected for a molecule of this size because of the duplicate resonances arising from the two isomers. In addition to the analyte resonances in Fig. 4, a number of other resonances associated with the separation media are also observed including the signal from the formic acid, the residual features from the suppression of the protonated acetonitrile (~2.0 ppm) and HO²H resonances (~4.7 ppm) and a multiplet corresponding to protons of the ammonium ion (6.9–7.2 ppm).

Utilizing a stop-flow HPLC–NMR experiment, a portion of the chromatographic peak for each isomer was isolated in turn in the NMR flow probe and spectra acquired to measure the isomerization rate. The chromatographic peaks in these experiments were much larger in volume than the 120 μl NMR flow cell, so only a portion of each peak was used in the NMR spectral analysis. Sequential chromatographic separations were performed to isolate each isomer individually due to the rapid equilibration rate. To maximize selectivity for the kinetic study, the front of the *s-trans* isomer peak and the tail of the *s-cis* isomer peak were selected for NMR analysis. However, the initial spectrum of the *s-cis* isomer contains *s-trans* isomer resonances as a minor component due to the chromatographic tailing of the *s-trans* isomer peak and the interconversion of these isomers on the chromatographic

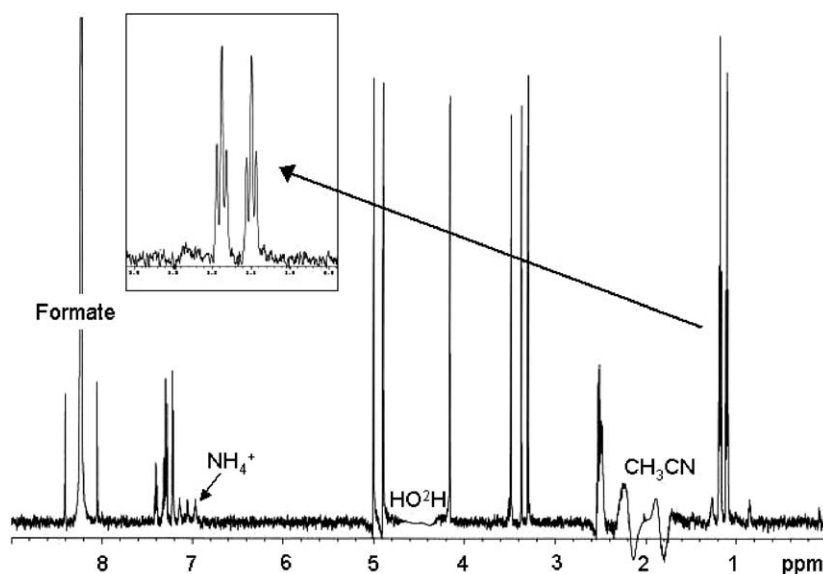


Fig. 4. The ¹H NMR spectrum of alachlor–ESA in CH₃CN (30%) and 25 mM ammonium formate buffer (70%) mobile phase system. The insert shows an expansion of the region of the spectrum containing the methyl protons resonances of the ethyl groups attached to the aromatic ring. Two methyl resonances are detected due to the presence of the *s-trans* and *s-cis* isomers of alachlor–ESA.

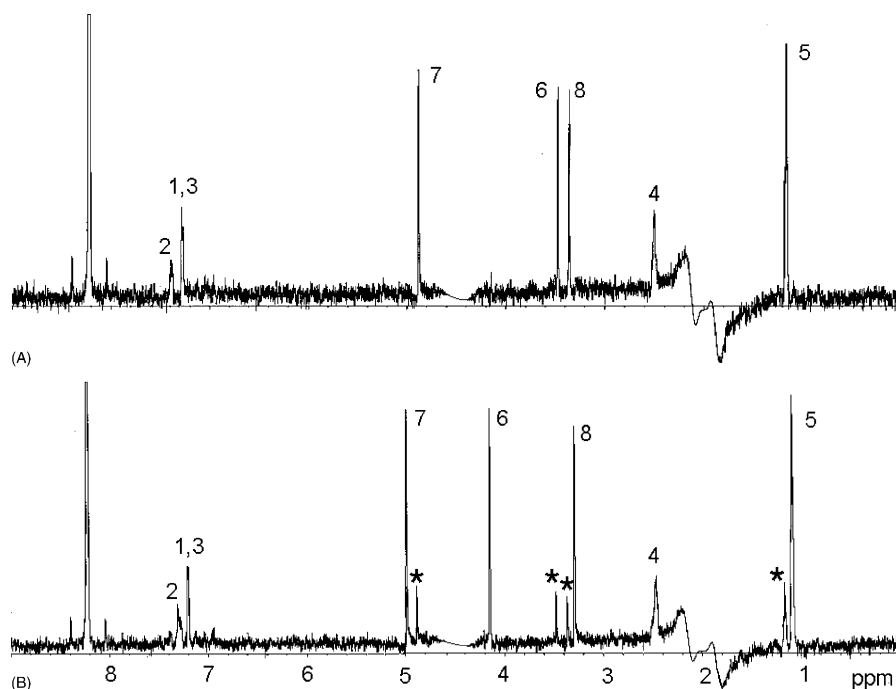


Fig. 5. Spectra of the separated alachlor-ESA *s-trans* (A) and *s-cis* (B) isomers. Because of tailing of the *s-trans* isomer peak in the chromatogram, the spectrum of the alachlor-ESA *s-cis* isomer contains some residual *s-trans* isomer resonances indicated by the asterisks (*).

time scale. The desired peak was selected at the UV detector and stopped in the active region of the NMR flow probe after a pre-calibrated transfer delay time. The NMR experiment was started immediately after the sample reached the flow cell. A series of ^1H NMR spectra were collected in set time intervals to follow the isomerization. The initial spectrum collected in each series contains primarily the proton resonances associated the isolated isomer of interest (Fig. 5) greatly simplifying the resonance assignment process for each isomer. Previous assignments of these proton resonances at equilibrium were difficult due to the complexity of the mixture.

The methyl resonances (Fig. 4, insert) were used to evaluate the isomerization rate of alachlor-ESA because they are well resolved and are the most intense peaks in the NMR spectrum. As shown in Fig. 6, by monitoring the intensity of the methyl resonances as a function of time the isomerization of the isolated *s-trans* isomer to the *s-cis* isomer can be followed. At equilibrium in this solvent system, the mixture contains essentially equal concentrations of the *s-trans* ($51 \pm 1\%$) and *s-cis* ($49 \pm 1\%$) isomers, a significantly higher fraction of the *s-trans* isomer than reported by previous authors for this and similar compounds in other solvents [16].

First order rate constants were determined by fitting a kinetic model based on a reversible first order reaction to the relative intensity data (Fig. 7). The integrals of the *s-trans* and *s-cis* isomer methyl resonances were converted to normalized intensities therefore, the sum of their normalized intensities equals one. The rate constants k_1 and k_{-1} for the isomerization for both the *s-trans* and *s-cis* alachlor-ESA were determined to be $2.0 \times 10^{-4} \pm 0.1 \times 10^{-4} \text{ s}^{-1}$ from

13 and 14 experimental trials, respectively. The free energy of activation (ΔG^\ddagger), 22.1 kcal/mol was determined from the rate constants using the approach described in by Larive and Rabenstein [12] (1 cal = 4.184 J). The similarity of the rate constants for the conversion of the two isomers is consistent with the value of the equilibrium constant, which is essentially one. In addition, the ΔG^\ddagger values for the isomerization of *s-cis* and *s-trans* alachlor-ESA are similar to values calculated for the *cis-trans* isomerization of various proline-containing peptides [12]. However, the rate constants determined in our experiments were larger by about a factor of 2 than those reported by Aga et al. for the isomerization of alachlor-ESA in $^2\text{H}_2\text{O}$ solution at 30°C [16]. Jayasundera et al. have used NMR to observe rapid exchange among the atropisomers of the acetanilide herbicide metolachlor in a variety of organic solvents including deuterated dimethyl sulfoxide (DMSO-d_6) [23]. The rate of conformational exchange of metolachlor atropisomers in DMSO-d_6 was observed to decrease when water ($^2\text{H}_2\text{O}$) was present [23]. The increased rate of isomerization in the presence of organic solvents such as DMSO and acetonitrile, the solvent utilized in these experiments, is consistent with the acceleration in organic solvents reported for the *cis-trans* isomerization of proline containing peptides [24]. In proline *cis-trans* isomerization, the proposed transition state is characterized by partial rotation of the C–N bond with no solvent participation and little solvent reorganization. In the twisted conformation proposed for the transition state, polar resonance structures for the amide bond are no longer possible, making the transition state less polar than the reactant [24]. The structural similarities between the

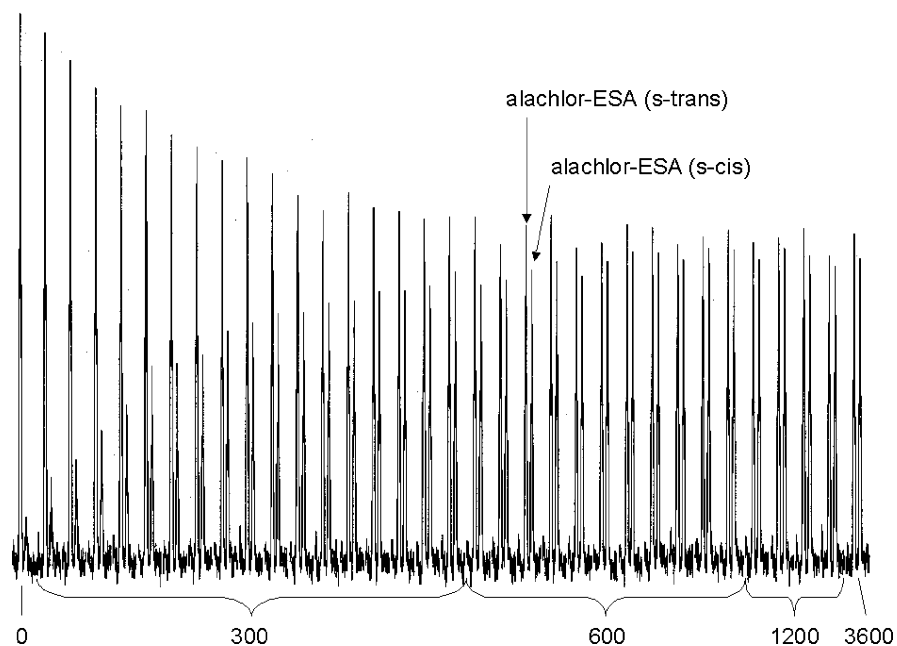


Fig. 6. Equilibration of the alachlor-ESA *s-trans* and *s-cis* isomers monitored by the ^1H NMR signal intensities of the methyl protons. The values of the pre-acquisition delay (seconds) used between each set of FIDs are shown below the spectra.

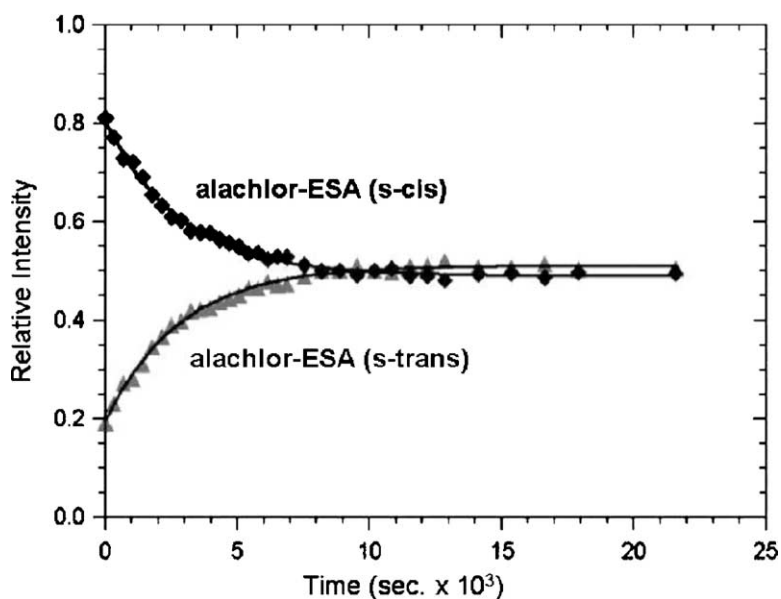


Fig. 7. Integrated resonance intensity of the *s-cis* (◆) and *s-trans* (▲) alachlor-ESA methyl peaks measured (at 20°C) as a function of equilibration time. The smooth lines through the points represent the theoretical curve calculated using nonlinear least-squares analysis of the data with a reversible first order kinetic model.

amide functional group in alachlor-ESA and the proline amide bond suggest a similar mechanism for the acceleration of isomerization of this compound in organic solvents.

4. Conclusion

The application of on-line HPLC-NMR afforded a simple and efficient means of evaluating the equilibrium associated

with the rotational isomers of alachlor-ESA. HPLC-NMR allowed the rapid detection of the ^1H NMR spectra of the separated isomers facilitating the resonance assignment process. The use of on-line detection allows for the analysis of reactions with faster rates than would be amenable with off-line analysis where time is lost in the collection of chromatographic fractions and delivering the sample to the detector. The use of a cryoflow probe, which has recently become commercially available, would allow for enhanced

sensitivity with an increase in signal-to-noise up to a factor of four when compared with a conventional NMR detection probe [25–27]. This increased sensitivity would in turn allow a decrease in the analyte concentration, which foralachlor–ESA would have improved the chromatographic resolution, or a decrease in the time required for signal averaging, increasing the rate with which NMR spectra could be acquired.

Acknowledgements

This research was supported by US Environmental Protection Agency grant R82900801-0. NSF grant DBI-0088931 and Kansas administered NSF EPSCoR partially funded the purchase of the 600 MHz NMR spectrometer used in this research.

References

- [1] E.M. Thurman, D.A. Goolsby, D.S. Aga, M.L. Pomes, M.T. Meyer, *Environ. Sci. Technol.* 30 (1996) 569.
- [2] M.D. Morton, F.H. Walters, D.S. Aga, E.M. Thurman, C.K. Larive, *J. Agric. Food Chem.* 45 (1997) 1240.
- [3] S.J. Kalkhoff, D.W. Koplín, E.M. Thurman, I. Ferrer, D. Barcelo, *Environ. Sci. Technol.* 32 (1998) 1738.
- [4] W.F. Schmidt, C.J. Hapeman, R.M. Waters, W.E. Johnson, *J. Agric. Food Chem.* 43 (1995) 1724.
- [5] S. Nesnow, S.C. Agarwal, W.T. Padgett, G.R. Lambert, P. Boone, A.M. Richard, *Chem. Res. Toxicol.* 8 (1995) 209.
- [6] H.-R. Buser, M.D. Müller, *Environ. Sci. Technol.* 29 (1995) 2023.
- [7] R.F.C. Brown, L. Radom, S. Sternhell, I.D. Rae, *Can. J. Chem.* 46 (1968) 2577.
- [8] M.D. Müller, H.-R. Buser, *Environ. Sci. Technol.* 29 (1995) 2031.
- [9] W.J. Wedemeyer, E. Welker, H.A. Scheraga, *Biochemistry* 41 (2002) 14637.
- [10] C. Dugave, *Curr. Org. Chem.* 6 (2002) 1397.
- [11] C. Schiene-Fischer, J. Habazettl, T. Tradler, G. Fischer, *Biol. Chem.* 383 (2002) 1865.
- [12] C.K. Larive, D.L. Rabenstein, *J. Am. Chem. Soc.* 115 (1993) 2833.
- [13] F. Thuncke, A. Kálmán, F. Kálmán, S. Ma, A.S. Rathore, Cs. Horváth, *J. Chromatogr. A* 744 (1996) 259.
- [14] S. Friebe, B. Hartrodt, K. Neubert, G.-J. Krauss, *J. Chromatogr. A* 661 (1994) 7.
- [15] S.A. Heberle, D.S. Aga, R. Hany, S.R. Müller, *Anal. Chem.* 72 (2000) 840.
- [16] D.S. Aga, S. Heberle, D. Rentsch, R. Hany, S.R. Müller, *Environ. Sci. Technol.* 33 (1999) 3462.
- [17] U.G. Sidelmann, C. Gavaghan, H.A.J. Carless, M. Spraul, M. Hofmann, J.C. Lindon, I.D. Wilson, J.K. Nicholson, *Anal. Chem.* 67 (1995) 4441.
- [18] U.G. Sidelmann, C. Gavaghan, H.A.J. Carless, R.D. Farrant, J.C. Lindon, I.D. Wilson, J.K. Nicholson, *Anal. Chem.* 67 (1995) 3401.
- [19] U.G. Sidelmann, S.H. Hansen, C. Gavaghan, H.A.J. Carless, J.C. Lindon, R.D. Farrant, I.D. Wilson, J.K. Nicholson, *Anal. Chem.* 68 (1996) 2564.
- [20] O. Corcoran, R.W. Mortensen, S.H. Hansen, J. Troke, J.K. Nicholson, *Chem. Res. Toxicol.* 14 (2001) 1363.
- [21] D.S. Aga, E.M. Thurman, M.E. Yockel, L.R. Zimmerman, T.D. Williams, *Environ. Sci. Technol.* 30 (1996) 592.
- [22] R.G. Bates, *pH and Other Acidity Functions for Nonaqueous Media, Determination of pH: Theory and Practice*, Wiley, New York, 1964, Chapter 8, p. 218.
- [23] S. Jayasundera, W.F. Schmidt, C.J. Hapeman, A. Torrents, *J. Agric. Food Chem.* 47 (1999) 4435.
- [24] R.K. Harrison, R.L. Stein, *J. Am. Chem. Soc.* 114 (1992) 3464.
- [25] P. Styles, N.F. Soffe, C.A. Scott, D.A. Cragg, F. Row, D.J. White, P.C.J. White, *J. Magn. Res.* 60 (1984) 397.
- [26] W.A. Anderson, W.W. Brey, A.L. Brooke, B. Cole, K.A. Delin, L.F. Fuks, H.D.W. Hill, M.E. Johanson, V.Y. Kotsubo, R.S. Withers, R. Nast, W.H. Wong, *Bull. Magn. Res.* 17 (1995) 98.
- [27] M. Spraul, A.S. Freund, R.E. Nast, R.S. Withers, W.E. Maas, O. Corcoran, *Anal. Chem.* 75 (2003) 1536.