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Kinetic analysis and subambient temperature on-line on-column derivatization of an active aldehyde

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Abstract

The chromatographic analysis of aldehydes under typical reversed-phase conditions may be a challenging task due to an equilibrium process leading to the formation of a gem diol species regardless of acidic or basic conditions. Initially, a reversed-phase HPLC gradient elution was developed to determine the amount of an acetylenic aldehyde in a reaction mixture. Significant fronting was observed under acidic and basic conditions even at −5 °C. In order to circumvent this problem, a reversed-phase HPLC gradient method on a C_{18} column at subambient temperature was developed using diethylamine as a mobile phase additive for on-line on-column derivatization of the aldehyde moiety. The on-line on-column reaction rate for the derivatization of the aldehyde with diethylamine was determined as a function of column temperature. An Arrhenius plot was constructed and the activation energy was calculated. The chromatographic behavior of the derivatized acetylenic aldehyde and products formed in-situ in the chromatographic system were studied at various temperatures ranging from −10 to 60 °C. It was found that the reaction products could be controlled by adjusting the column temperature. Different reaction pathways were identified as a function of temperature. The products and the reaction pathways were characterized by NMR, LC–MS and UV spectra.

Keywords: Derivatization, LC; Activation energy; Arrhenius plot; Gem–diol formation; Aldehydes

1. Introduction

Peak shape optimization is essential during HPLC method development [1–3]. Peak distortion such as fronting or tailing can cause poor efficiency and low resolution between solutes. Peak tailing can arise from several sources including sample overload and analyte interaction with residual silanols. Peak fronting can arise from sample diluent/mobile phase mismatch [4], chemical ionic equilibrium [5], and on-column reactions [6,7]. The kinetics of on-column reactions can be controlled by optimization of the temperature, pH [8], flow-rate [9] and addition of mobile phase additives.

Peak distortion has been observed during the reversed-phased HPLC analysis of enalapril [8] and captopril [10–13], angiotensin-converting enzyme

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(ACE) inhibitors. Enalapril exists as a mixture of two conformers in aqueous solution. This is due to the amide *cis–trans* isomerization of the proline peptide. Reversed-phase HPLC had shown two unresolved peaks as a result of this conformational change with slow kinetics. It was shown that the two peaks coalesce upon decreasing the flow-rate and increasing the temperature.

Aldehydes and ketones are weak Brønsted acids and are susceptible to addition reactions at electrophilic carbon atoms. In the presence of suitable reagents this can lead to keto–enol tautomerization, which chromatographically is shown as fronted peaks [6].

Severe peak fronting was observed for oxycodone, a compound containing a carbonyl moiety, employing reversed-phase chromatography under basic conditions using a methanol–phosphate buffer mobile phase on a C_{18} column [14]. Using deuterium-labeled oxycodone and analysis by high-performance liquid chromatography–mass spectrometry (LC–MS) it was shown that the formation of a gem–diol and a hemiketal of oxycodone are a result of the addition of water and methanol, respectively, to the ketone moiety on oxycodone. By decreasing the temperature to 0 °C, the rates of interconversion were decreased, allowing for the separation of these species from each other and from oxycodone. Upon increasing the temperature to 60 °C, the rates of interconversion increased and the three species eluted as a single peak with improved peak symmetry.

The column temperature will influence the distribution coefficient and kinetics of an equilibrium/interconversion process. Physico-chemical information can be obtained from liquid chromatography [15]. Determining the kinetics of these reactions may help to determine the best chromatographic conditions in order to obtain good chromatographic peak shapes.

The on-column chemical reaction can be controlled as a function of temperature and mobile phase conditions. For instance, the inherent instability of active esters led to a chemical reaction during the chromatographic process that gave rise to distorted peaks which under protic conditions underwent intramolecular ring closure to a cyclic ether [16]. The on-column cyclization was found to be extensive at room temperature. However, kinetic analysis justified low-temperature chromatography, making the on-column cyclization insignificant. Henderson and Mello [7] and Henderson and Horváth [17] showed that lowering the temperature of separation for proline-based peptides under reversed-phase conditions retarded *cis–trans* isomerization of the amide group and enhanced their separation.

Derivatization, when possible, is a viable alternative to stop such reactions and improve peak shape. For example, derivatization of aldehydes prior to chromatographic analysis is an alternative to prevent potential interaction with mobile phase components [18,19]. On-line on-column derivatization with amines could also be used to suppress the reaction of the aldehyde with the mobile phase components. Amines are an important class of derivatizing reagents since they can behave as nucleophiles toward the carbonyl group of the aldehyde. Primary amines react with aldehydes forming Schiff bases while the reaction with secondary amines yields secondary carbinolamines [20].

In this paper, we report the chromatographic separation of an acetylenic aldehyde whose carbonyl moiety under acidic or basic conditions promotes the formation of a gem–diol species leading to a severely fronting peak. On-line on-column derivatization of the acetylenic aldehyde with diethylamine as a mobile phase additive was employed to suppress the formation of these species. To our knowledge this is the first example of an on-line on-column derivatization of an active aldehyde with an amine mobile phase additive. The rate of reaction between the acetylenic aldehyde and diethylamine was determined at several temperatures and the results were used for optimization of the chromatography.

2. Experimental

2.1. Chemicals and reagents

Solvents: Tetrahydrofuran (THF), triethylamine (TEA) and trifluoroacetic acid (TFA) were purchased from Sigma–Aldrich (St. Louis, MO, USA), acetonitrile HPLC grade from EM Science (Gibbstown, NJ,

USA), diethylamine (DEA) 99.5% redistilled from Aldrich (Milwaukee, WI, USA), and phosphoric acid from Fluka (Messerschmittstr, Germany). HPLC-grade water was generated by a Milli-Q water system (Millipore, Bedford, MA, USA).

2.2. Chromatography and LC–MS apparatus

An Agilent HP1100 HPLC system with a diode array detector was employed. For all temperature analyses the column was placed in a column jacket (Aura Industries, Staten Island, NY, USA) containing a thermometer and connected to a constant-temperature bath (Neslab Instruments, Newington, NH, USA) with recirculating ethylene glycol. Chromatograms were processed using a PE Nelson version 3.1 data acquisition system (Cupertino, CA, USA).

The HPLC system used for the LC–MS experiments was the same as used for the chromatography experiments and was interfaced either to an Agilent HP1100 Series LC–MSD (single quadrupole) or Finnigan TSQ 7000 instrument. Atmospheric pressure ionization using the electrospray interface operated in the positive ion mode was used for the MS investigations. The electrospray needle for the LC–MSD experiments was maintained at 4.0 kV, with drying gas flow at 12 L/min, nebulizer pressure at 50 p.s.i., drying gas temperature operated between 250 and 350 °C, and fragmentor voltage at 80 V. For the TSQ experiments, the instrument was operated in full-scan mode using the first quadrupole (Q1) as the mass analyzer and the second (Q2) and third (Q3) quadrupoles as transmission devices. The spray voltage was 4.5 kV. The desolvation gas was nitrogen with the sheath and auxiliary settings at 60 p.s.i. and 30 units (rotometer), respectively. The interface capillary was operated between 200 and 250 °C. The manifold was set at 70 °C. The photomultiplier was operated at 1.25 kV. The scan range was from 300 to 900 amu at a dwell time of 1.67 ms. The mass spectrometer was operated at unit resolution. In the product ion (MS–MS) mode, the collision gas was argon set at a cell pressure of 2.0 mTorr and the collision energy was −25 eV. The resolution on the first quadrupole was decreased to 3.0 amu full width half height for the precursor ion to increase sensitivity.

2.3. Chromatography conditions

A Hypersil BDS-C_{18} 25×0.46 cm I.D. column (Agilent, Wilmington, DE, USA) was used with a 20 min linear gradient at a flow-rate of 1 mL/min from 40:60 to 10:90 (A–B, v/v) where B consisted of 3% (v/v) THF in acetonitrile and A consisted of the aqueous portion of the mobile phase. The aqueous portion of the mobile phase was either 0.1% (v/v) trifluoroacetic acid (TFA) or 0.1% (v/v) triethylamine (TEA) adjusted to pH 7.5 with TFA for the initial experiments. For the on-line on-column derivatization experiments the aqueous portion of the mobile phase contained 0.2% (v/v) diethylamine (DEA) and the final pH was adjusted to 7.0 with phosphoric acid. The pH was measured before the addition of the organic component using a Fisher Scientific AR50 pH meter. The analytes were dissolved in acetonitrile at a concentration of 0.5 mg/mL and 10 μL was injected into the HPLC system. Compounds were detected by a UV photodiode array detector set to a wavelength of either 220 or 320 nm.

2.4. LC–MS conditions

The same gradient used for the chromatography experiments was employed. Three different mobile phases were used for LC–MS. The aqueous portion of the eluent used for the LC–MS experiments consisted of: (1) 0.1% (v/v) TFA; (2) 0.1% (v/v) TEA adjusted to pH 7.5 with TFA and with post-column addition of TFA (40%, v/v); (3) 0.2% (v/v) DEA adjusted to pH 7.0 with H_3PO_4 with post-column addition of TFA (40%, v/v). The addition of TFA post-column lowered the effluent pH and was used to improve the sensitivity.

2.5. NMR experimental

Proton and carbon-13 spectra were recorded in CD_3CN on a Bruker AMX400 at a frequency of 399.87 and 100.56 MHz, respectively. The chemical shifts are reported in ppm relative to residual CHD_2CN for proton ($\delta = 1.93$ ppm) and CD_3CN for carbon ($\delta = 1.3$ ppm). All coupling constants are reported in Hertz (Hz) with proton multiplicities abbreviated as follows: s, singlet; d, doublet; t,

triplet; q, quartet; m, multiplet; br, broad; o, overlapping.

2.5.1. Acetylenic aldehyde

^{1}H NMR δ 9.13 (s, 1H), 7.75 (s, 1H), 7.38 (br, 2H), 7.29 (s, 2H), 7.06 (br s, 2H), 4.88 (q, $J = 6.7$, 1H), 4.32 (d, $J = 2.8$, 1H), 4.22 (td, $J = 11.5$, 2.8, 1H), 3.65 (ddd, $J = 11.4$, 3.6, 1.6, 1H), 3.57 (d, $J = 2.8$, 1H), 3.48 (d, $J = 18.5$, 1H), 3.36 (d, $J = 18.5$, 1H), 2.88 (br d, $J = 11.5$, 1H), 2.79 (td, $J = 11.5$, 3.6, 1H), 1.39 (d, $J = 6.7$, 3H).

^{13}C NMR δ 178.6, 163.2 (d, $J_{CF} = 244.1$), 147.0, 133.2 (d, $J_{CF} = 3.2$), 132.2 (d, $J_{CF} = 8.8$, 2C), 131.5 (q, $J_{CF} = 32.9$, 2C), 127.6 (m, 2C), 124.1 (q, $J_{CF} = 272.2$, 2C), 122.1 (m), 115.6 (d, $J_{CF} = 22.5$, 2C), 95.9, 91.1, 86.7, 72.6, 67.0, 59.5, 51.8, 44.2, 24.4

2.5.2. Michael adduct

^{1}H NMR δ 9.56 (d, $J = 8.4$, 1H), 7.78 (m, 1H), 7.54 (br, 2H), 7.40 (s, 2H), 7.08 (br t, $J = 8.4$, 2H), 5.09 (d, $J = 8.4$, 1H), 4.91 (q, $J = 6.6$, 1H), 4.30 (d, $J = 2.4$, 1H), 4.14 (td, $J = 11.4$, 2.4, 1H), 3.60 (om, 1H), 3.59 (ddd, $J = 11.4$, 3.6, 1.6, 1H), 3.57 (om, 1H), 3.37 (od, $J = 13.7$, 1H), 3.37 (d, $J = 2.8$, 1H), 3.33 (om, 1H), 3.23 (om, 2H), 3.04 (d, $J = 13.7$, 1H), 2.84 (dt, $J = 11.6$, 2.0, 1H), 1.43 (d, $J = 6.8$, 3H), 1.20 (br t, $J = 6.8$, 3H), 1.06 (br t, $J = 6.8$, 3H).

^{13}C NMR δ 186.6, 163.4 (d, $J_{CF} = 244.1$), 160.8, 147.0, 133.8 (d, $J_{CF} = 2.4$), 132.6 (d, $J_{CF} = 8.0$, 2C), 131.5 (q, $J_{CF} = 32.9$, 2C), 127.7 (m, 2C), 124.1 (q, $J_{CF} = 272.2$, 2C), 122.1 (m), 115.5 (d, $J_{CF} = 21.7$, 2C), 103.9 (br), 95.7, 72.4, 71.4, 59.9, 52.0, 51.6, 45.5, 44.3, 24.4, 14.2, 11.1.

2.5.3. Aminal

^{1}H NMR δ 7.75 (s, 1H), 7.54 (br, 2H), 7.28 (s, 2H), 7.08 (br t, $J = 8.4$, 2H), 4.87 (q, $J = 6.8$, 1H), 4.30 (od, 1H), 4.20 (td, $J = 11.4$, 2.8, 1H), 3.99 (t, $J = 1.6$, 1H), 3.62 (ddd, $J = 11.4$, 3.6, 1.6, 1H), 3.58 (od, 1H), 3.24 (t, $J = 1.6$, 2H), 2.84 (om, 1H), 2.67 (m, 4H), 2.51 (om, 4H), 2.41 (om, 1H), 1.38 (d, $J = 6.8$, 3H), 0.91 (td, $J = 7.2$, 2.0, 12H).

^{13}C NMR δ 163.1 (d, $J_{CF} = 244.1$), 147.1, 133.7 (d, $J_{CF} = 2.4$), 132.6 (d, $J_{CF} = 8.0$, 2C), 131.5 (q, $J_{CF} = 32.9$, 2C), 127.5 (m, 2C), 124.1 (q, $J_{CF} = 272.2$, 2C), 122.1 (m), 115.5 (d, $J_{CF} = 21.7$, 2C), 96.1, 82.8, 79.4, 72.5, 71.9, 66.8, 59.6, 51.6, 43.9, 43.1 (4C), 24.4, 12.6_3 (2C), 12.6_0 (2C).

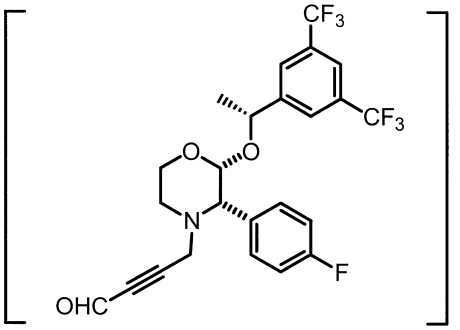
2.5.4. Secondary carbinolamine

^{1}H NMR δ 7.76 (s, 1H), 7.66 (br m, 2H), 7.25 (s, 2H), 7.17 (br m, 1H), 6.92 (br m, 1H), 5.68 (br s, 1H), 5.02 (d, $J = 14.9$, 1H), 4.86 (q, $J = 6.4$, 1H), 4.27 (d, $J = 2.40$, 1H), 4.17 (td, $J = 10.8$, 4.4, 1H), 3.61 (d, $J = 10.8$, 1H), 3.53 (t, $J = 3.2$, 1H), 3.26 (d, $J = 17.3$, 1H), 3.18 (d, $J = 17.3$, 1H), 2.78 (om, 2H), 2.62 (m, 2H), 2.51–2.43 (om, 2H), 1.35 (d, $J = 6.4$, 3H), 0.98 (ot, $J = 7.2$, 3H), 0.97 (ot, $J = 7.2$, 3H).

^{13}C NMR δ 162.9 (d, $J_{CF} = 243.3$), 146.9, 133.4, 132.2 (m), 131.4 (m), 131.10 (q, $J_{CF} = 32.9$, 2C), 127.4 (m, 2C), 123.9 (d, $J_{CF} = 272.2$, 2C), 122.0 (m), 115.7 (d, $J_{CF} = 24.1$), 114.9 (d, $J_{CF} = 21.7$), 95.7, 84.2, 77.8, 75.8 (d, $J = 3.2$), 72.1, 66.5, 59.3, 51.3, 43.6, 43.0 (2C), 24.3, 13.3 (2C).

3. Results and discussion

Acetylenic aldehyde (Fig. 1) is used as an intermediate in the synthesis of an active pharmaceutical ingredient. From a process/synthetic perspective, an accurate quantitation of the acetylenic aldehyde compound was needed to establish the proper amounts of reagents charged in the next step of the synthesis. Initially, the acetylenic aldehyde was analyzed at ambient temperature using reversed-phase chromatography and gradient conditions, under acidic [0.1% (v/v) TFA] (Fig. 2) and basic



MW 503.42

Fig. 1. Structure of acetylenic aldehyde.

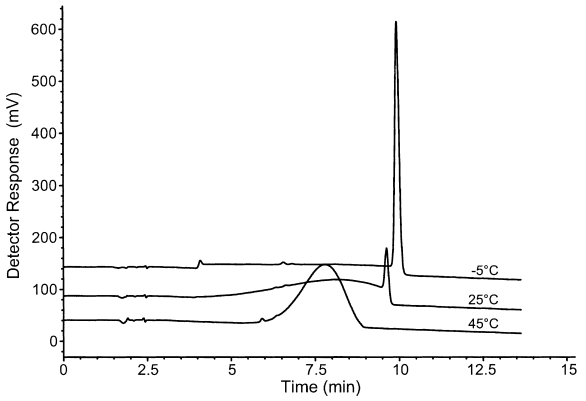


Fig. 2. Influence of temperature on the formation of the gem–diol species. HPLC conditions: column, HP BDS-C_{18}-Hypersil, 25 cm×4.6 mm, 5 μm, 100 Å; temperature, −5, 25, 45 °C; mobile phase, A—0.1% (v/v) TFA, B—3% (v/v) THF in acetonitrile; elution conditions, 20 min gradient from 60% B to 90% B; wavelength, 220 nm; flow-rate, 1.0 mL/min.

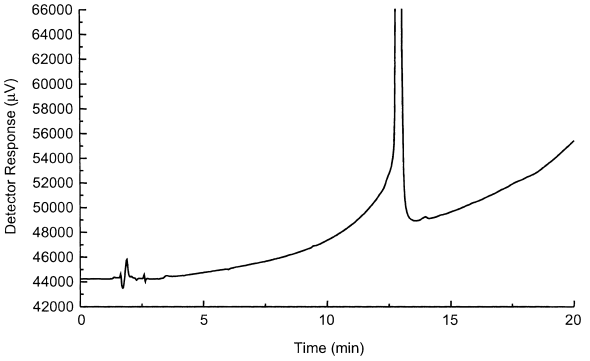


Fig. 3. Acetylenic aldehyde analyzed at 20 °C employing on-column derivatization with diethyl amine. HPLC conditions: column, Agilent BDS-C_{18}-Hypersil, 25 cm×4.6 mm, 5 μm, 100 Å; temperature, 20 °C; mobile phase, A—0.2% (v/v) DEA adjusted to pH 7.0 with phosphoric acid, B—3% (v/v) THF in acetonitrile; elution conditions, 20 min gradient from 60% B to 90% B; wavelength, 220 nm; flow-rate, 1.0 mL/min.

conditions [0.1% (v/v) TEA adjusted to pH 7.5 with TFA]. In both cases, severe peak fronting was observed. Aldehydes and ketones are known to form a gem–diol under both acidic and basic conditions [20]. The reaction is an equilibrium and chromatographically can manifest as fronted peaks. The chromatogram of Fig. 2 suggests the existence of equilibrium species regardless of the temperature, presumably attributed to gem–diol formation. Evidence for this will be given below.

Initial optimization experiments carried out at temperatures ranging from −5 to 45 °C under basic or acidic conditions did not eliminate peak fronting. Therefore, in order to circumvent the formation of the gem–diol, derivatization of the aldehyde became a viable alternative. Diethylamine was added to the mobile phase, with the aim of tagging the aldehyde and yielding an improvement in the peak shape. An on-column reaction has an advantage since it does not require any sample preparation and the desired acetylenic compound derivative could be formed in situ. A chromatogram at 20 °C employing on-column derivatization with diethylamine is shown in Fig. 3. Fronting was observed, indicating an on-column chemical reaction.

The reaction of aldehydes with secondary amines is temperature dependent and throughout the reaction several reaction products are formed. Therefore, the separation using the diethylamine mobile phase additive was carried out at various temperatures from −10 to 60 °C and the peaks were detected using photodiode array and mass spectrometric detection. Each reaction product was followed and confirmed using LC–MS and NMR and the reaction pathways were identified.

3.1. Temperature effects on chromatographic peak shapes

Using diethylamine in the mobile phase the chromatography experiments were conducted at temperatures ranging from −10 to 60 °C. Chromatographic overlays at the different temperatures monitored at 220 and 320 nm are presented in Figs. 4 and 5, respectively. One sharp distinct peak was observed at −10 °C. The photodiode array spectra at three points across the peak were superimposable only at −10 °C (Fig. 6a) showing that the peak was chromatographically and spectrally homogenous. As the temperature was increased from 0 to 60 °C fronting was observed and the peak maximum was shifted to shorter retention times. The increase in temperature to 60 °C also led to the formation of a later eluting peak.

The diode array spectra at three points across the peak eluting at 10.3 min were not superimposable at 20 °C (Fig. 6b), indicating peak heterogeneity. From the photodiode array spectra a maxima in the region

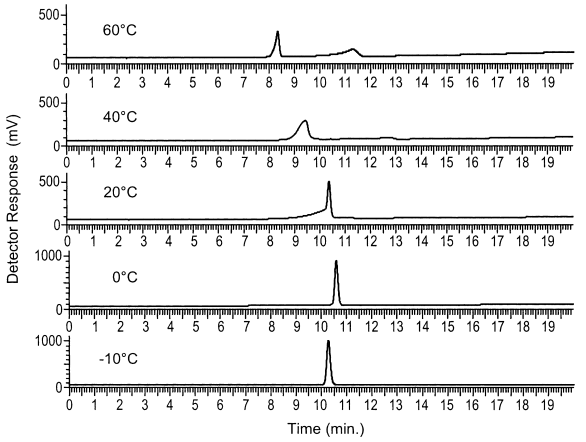


Fig. 4. Influence of temperature on the formation of the reaction products analyzed at 220 nm. HPLC conditions as in Fig. 3, with variable temperature from −10 to 60 °C.

of 260–270 nm in the front of the peak was observed corresponding to the aromatic portion of the molecule. New maxima in the region between 300 and 360 nm at the apex and back side of the peak were also observed. This new wavelength maximum is representative of a compound with higher conjugation in the molecule [21]. The observed decrease of retention time with increase in temperature may be attributed to the formation of new chemical species and not to a decrease of the distribution coefficient.

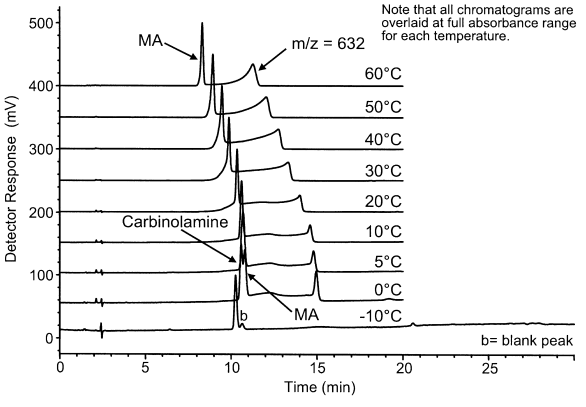


Fig. 5. Influence of temperature on the formation of the reaction products analyzed at 320 nm. HPLC conditions as in Fig. 3, with variable temperature from −10 to 60 °C.

3.2. LC–MS

To ascertain the identity of the species being formed on-column under the mobile phase conditions with and without diethylamine, LC–MS was performed. Atmospheric pressure ionization using the electrospray interface operated in the positive ion mode was used for MS and MS–MS investigations. Full-scan mass spectra were obtained from 150 to 1000 amu and the species were determined as quaternary salts or as pseudo-molecular ions $[M+H]^+$.

LC–MS was performed at −5, 25 and 45 °C under acidic conditions [0.1% (v/v) TFA] and basic conditions [0.1% (v/v) TEA as the mobile phase modifier with TFA post-column addition] using the same gradient as for the initial chromatography described in the Experimental section. Mass spectra at the apex of the peak obtained at −5 °C showed m/z 504 and 545, corresponding to the protonated molecule and acetonitrile adduct of acetylenic aldehyde, respectively. Mass spectra taken across the peak front indicated a m/z 522 species assigned as the acetylenic aldehyde's gem–diol. Similar mass spectra were obtained at 25 and 45 °C.

LC–MS experiments were performed to mimic the conditions in HPLC using DEA as mobile phase modifier. Full-scan mass spectra (Fig. 7) were taken at peak apex (Fig. 4) during the temperature study (−10, 1, 25, 50 °C) and distinct differences were observed. At −10 °C the predominant species was m/z 577 with a smaller amount of the m/z 632 species. At 1 °C the predominant species was m/z 504, with smaller amounts of m/z 522, 577 and 632. At 25 and 50 °C the predominant species were m/z 577 and 632, with a smaller amount of m/z 504 observed at 25 °C only.

To ascertain whether these species were artifacts of the system the effect of the capillary drying gas temperature between 250 and 350 °C was examined at a column temperature of −10 °C. The dynamic ionization process using electrospray involves nebulization with progressive solvent removal from the charged particle clusters, and the solvent may react with an intermediate species during the course of the ionization process, thus perturbing the reaction pathway. The intensity of the m/z 632 species was found to increase with increasing drying gas tem-

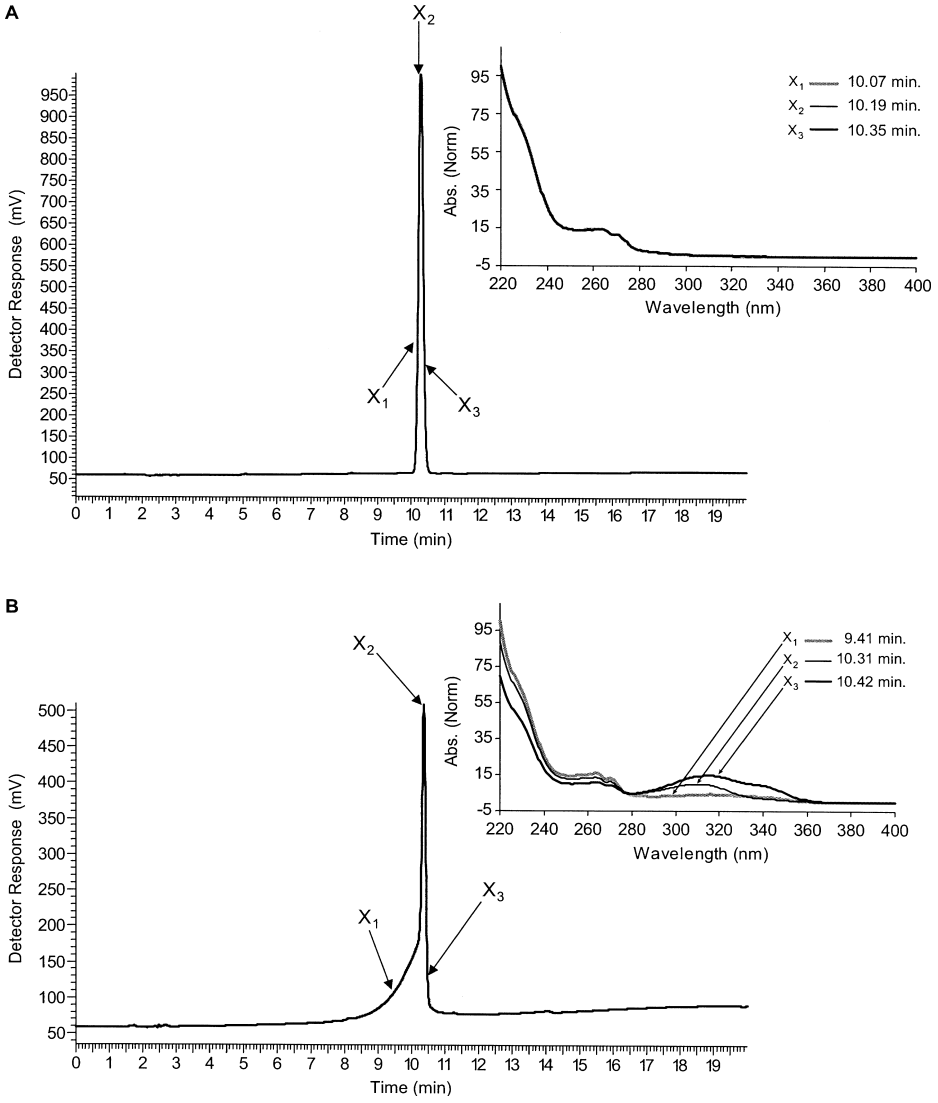


Fig. 6. (a) HPLC chromatogram and superimposition of diode array spectra of acetylenic aldehyde analyzed at −10 °C. HPLC conditions as in Fig. 3. (b) HPLC chromatogram and superimposition of diode array spectra of acetylenic aldehyde analyzed at 20 °C. HPLC conditions as in Fig. 3.

perature with concomitant formation of m/z 559 species. The optimal capillary drying gas temperature was found to be 250 °C. Moreover a m/z 246 species was observed when the temperature of the HPLC system was held at 1°C and 25°C. This species was presumed to be a rearrangement species of the m/z 504 species (Acetylenic aldehyde) and deemed to be an artifact of the system. This was confirmed by NMR since a species consistent with m/z 246 was not observed in any of the experiments.

3.3. NMR

The addition of DEA could lead to the formation of multiple species. NMR was used for the elucidation of their structures (Fig. 8). The NMR experi-

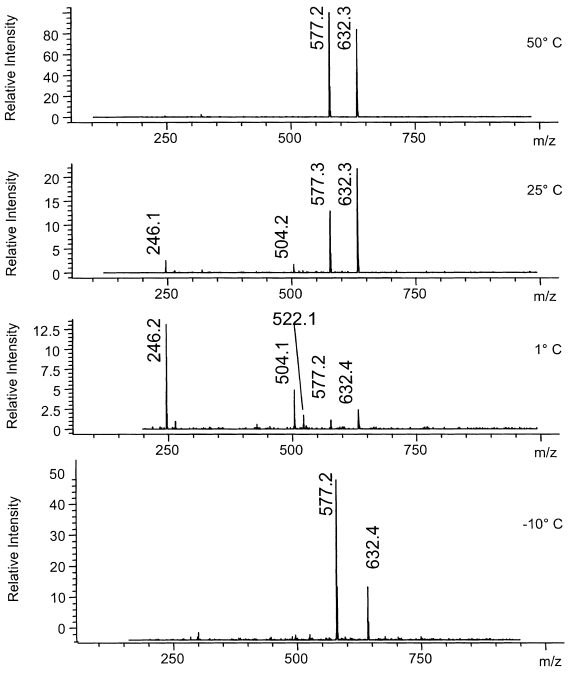


Fig. 7. Effect of temperature on the reaction products formed in situ. HPLC elution conditions as in Fig. 3 at different temperatures (−10, 1, 25, 50 °C). Mobile phase, A—0.2% (v/v) DEA adjusted to pH 7.0 with phosphoric acid, B—3% (v/v) THF in acetonitrile. Atmospheric pressure ionization using the electrospray interface operated in the positive ion mode was used for the MS investigation. Full-scan mass spectra were obtained in the mass range from 100 to 1000 amu and the species were determined as their pseudo-molecular ions $[M+H]^+$. Agilent HP1100 Series LC–MSD conditions: drying gas flow, 12 L/min; nebulizer pressure, 50 p.s.i.; drying gas temperature, 250 °C; capillary voltage, 4 kV; fragmentor voltage, 150 V. Post-column ionization was performed with 40% (v/v) TFA in water. This reagent solution was pumped at 20 μL/min using a Harvard Infusion pump and was mixed with the HPLC effluent using a T connector just prior to MS.

ments were run using diluent solutions similar to the mobile phase composition used in HPLC experiments with DEA as additive. The reaction of acetylenic aldehyde **1** with six equivalents of DEA and water in deuterated acetonitrile was monitored at temperatures of 60, 27, 0, −20 and at −40 °C. At low temperatures the main product observed was the secondary carbinolamine **2**, which was consistent with m/z 577 observed in the LC–MS experiments. During the NMR experiments, conversion to the carbinolamine was rapid at 0, −20 and −40 °C and was found to subsequently rearrange through the acetylenic aldehyde **1** to form a diethylamine Michael adduct **5**, a m/z 577 species, at a rate that increased with increasing temperature. Full conversion of the carbinolamine at −20 °C took approximately 2 h. The presence of a small but increasing amount (4–7 mol%) of an aminal **6** was formed in all three low-temperature reactions. The lower amount was formed at −40 °C. This is consistent with the LC–MS data that yielded m/z 632 amu for the aminal at low temperatures. As the temperature in the NMR experiments was increased from 0 to 27 °C there was a decrease in the amount of aminal **6**. A concomitant increase of a diethylamine iminium Michael adduct (IMA) (**4**) was observed. The presence of IMA was confirmed by spiking with an authentic sample of IMA. From the LC–MS data shown in Fig. 9, the extracted ion mass chromatogram at 1 °C of m/z 632 also shows two distinct species. As the temperature was increased to 60 °C the formation of the IMA was seen to increase while the aminal was decreasing.

However, when only 1.2 equivalents of DEA (−40 to 0 °C) were used, only the aminal **6** was observed in NMR spectra and the formation of IMA (**4**) at higher temperatures (0–60 °C) was not detected, indicating an excess of DEA is needed to promote the generation of IMA. As the temperature was increased the amount of carbinolamine **2** decreased and the diethylamine Michael adduct **5** increased. At 60 °C, the diethylamine Michael adduct **5** was the predominant species and appears stable with minimal degradation.

In all reactions, using 6 or 1.2 equivalents DEA, NMR spectra were conclusively able to distinguish between the aminal **6** and IMA **4** (m/z 632 species) and between the carbinolamine **2** and the diethylamine Michael adduct **5** (m/z 577 species), both of which were indistinguishable from their mass spectra. The fragmentation pathway for this class of compounds is scission at the morpholine nitrogen–carbon side chain bond; the resulting product ion spectra are not unique and unequivocal structural assignments could not be made. Additionally, LC–MS experiments using alkali salts as post-column additives to distinguish the aminal (**6**) from IMA (**4**) were unsuccessful as no $[M+X]^+$ ions (X = Na, K, Li) were observed for **6**.

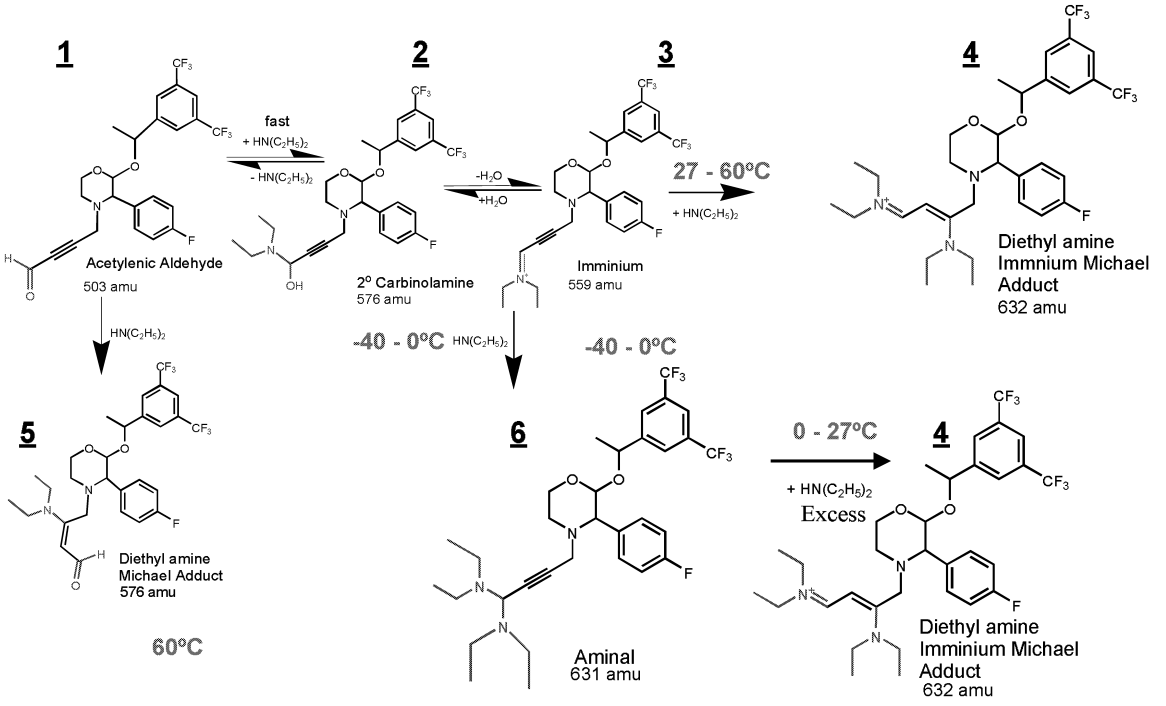


Fig. 8. Proposed pathways for the reaction of acetylenic aldehyde with diethylamine.

Based on the LC–MS and NMR data the reaction schemes shown in Fig. 8 were proposed for the on-line derivatization of the aldehyde with diethylamine. The diethylamine in the mobile phase adds to the carbon–oxygen double bond of the acetylenic aldehyde **1** in an addition step that leads to the formation of a secondary carbinolamine compound **2**, which is proposed to be in equilibrium with acetylenic aldehyde **1**. The secondary carbinolamine **2** may undergo an elimination step (removal of water) to form an imminium compound **3**. These two species are proposed to be in equilibrium. However, another possible pathway suggests diethylamine addition to the carbon–carbon triple bond of **3**, which could lead to the formation of IMA **4**, or to the alpha carbon, leading to the formation of the aminal **6**.

Another pathway suggests the diethylamine adding to the carbon–carbon triple bond of the acetylenic aldehyde **1** through nucleophilic addition. This would lead to the formation of the diethylamine Michael adduct **5** [22]. Based upon the MS (Figs. 7 and 9) and MS–MS data it was not possible to unequivocally discern the structure for the two m/z 577 species since the fragmentation patterns for the diethylamine Michael adduct **5** and carbinolamine **2** species were deemed to be indistinguishable. Also, the m/z 632 species, aminal **6** and IMA **4**, were indistinguishable from their mass spectra. Therefore, the identification of products obtained at different temperatures was supported by ^{1}H and ^{13}C NMR.

At all temperatures studied (Figs. 4 and 5), LC spiking experiments with an authentic sample of IMA were performed and it was confirmed that the retention of this species did not coelute with that of the secondary carbinolamine and diethylamine Michael adduct species. The later eluting peak at increasing temperatures was thus determined to be IMA. Spiking experiments at various temperatures with a synthesized sample of diethylamine Michael adduct were also performed and its retention time was confirmed at the different temperatures. It was also shown that the diode array spectra at three points across the peak were not superimposable at 20 °C (Fig. 6b), indicating the presence of multiple species in the same peak. From the photodiode array experiments it was shown that the back of the peak

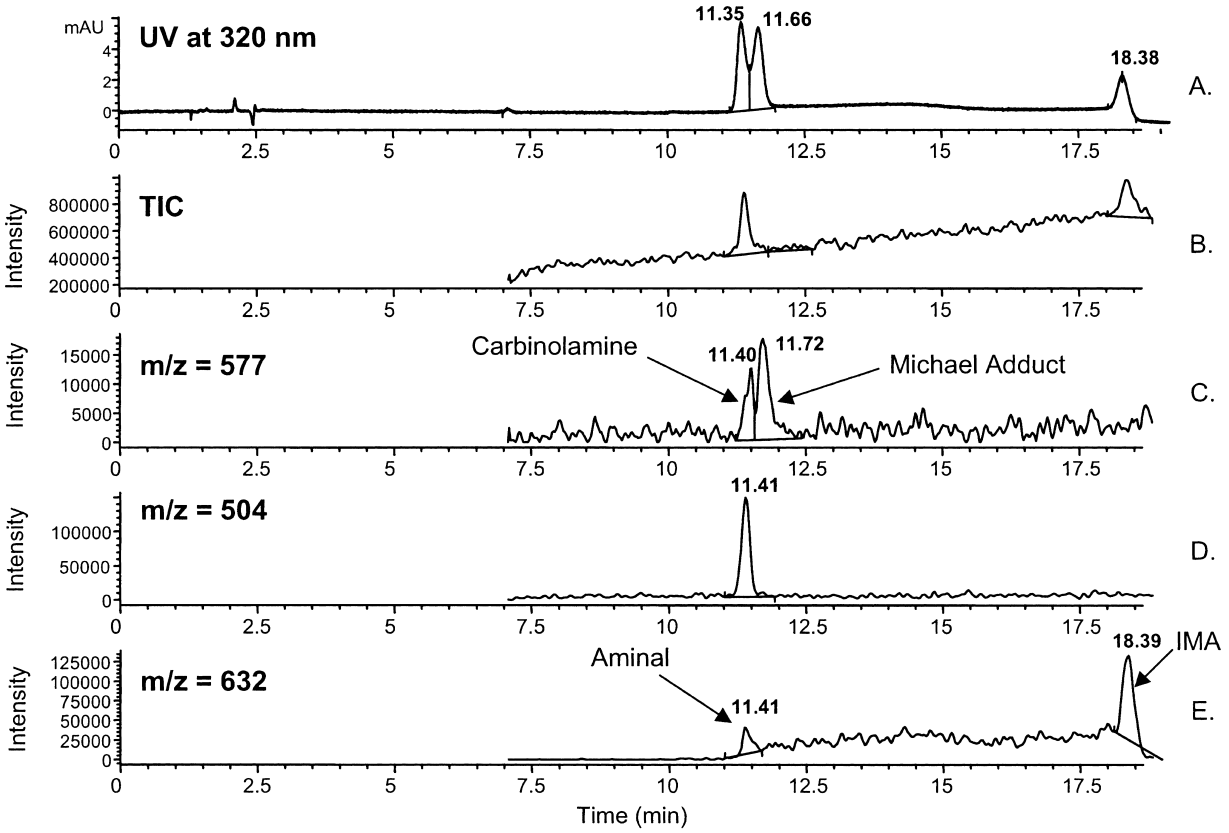


Fig. 9. Total ion chromatogram and extracted ion chromatograms at 1 °C for acetylenic aldehyde. HPLC and LC–MS conditions as in Fig. 7. (A) UV profile at 320 nm. (B) Total ion chromatogram. (C) Extracted ion chromatogram, $m/z=577$. (D) Extracted ion chromatogram, $m/z=504$. (E) Extracted ion chromatogram, $m/z=632$.

contained species that had a wavelength maxima at a longer wavelength. This was indicative of the carbinolamine rearranging to form the diethylamine Michael adduct, a more conjugated species. At the same time, the diode array spectra at the front and apex of the peak analyzed at 60 °C were superimposable with that of an isolated sample of diethylamine Michael adduct.

Therefore, the NMR, LC–MS and diode array spectra conclusively confirmed that, at low temperature, the acetylenic aldehyde, in the presence of excess DEA, exists as the carbinolamine and, at higher temperatures, converts to the more thermodynamically stable diethylamine Michael adduct.

3.4. Kinetics study

The fronting observed when the acetylenic aldehyde was analyzed at temperatures ranging from 20 to 40 °C in the presence of DEA is presumably consistent with the formation of products during the chromatographic analysis. Each point on the main peak is proportional to the amount of aldehyde that reacted with the DEA. In order to estimate the kinetics of the reaction between the acetylenic aldehyde and DEA, the chromatogram was baseline corrected. An estimated baseline was drawn from the beginning of the front of peak 1 to the end of the peak. Perpendiculars (h_i) on the baseline were drawn at regular time intervals [16]. The h_i's were considered to be proportional to the amount of aldehyde [*A*] which disappears through the reaction with DEA on the column at the corresponding retention time. The retention time and reaction time scales were assumed to be equal. Since diethylamine was in large excess the amount of diethylamine that disappears

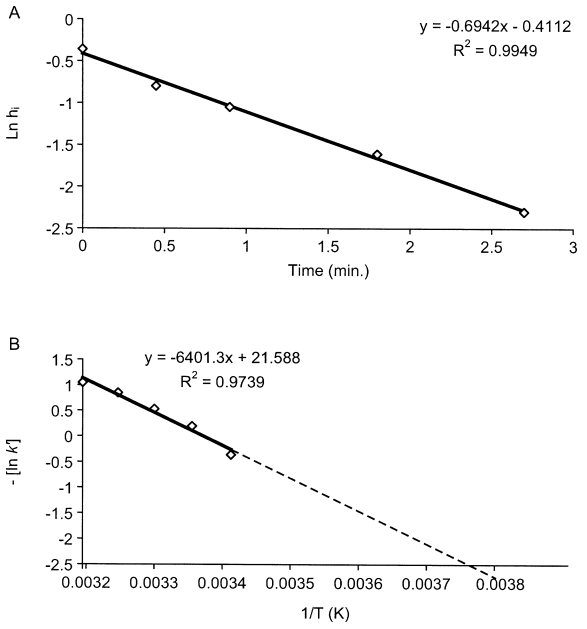


Fig. 10. (a) Kinetic plot for the rate constant determined at 20 °C. (b) Arrhenius plot for aldehyde conversion.

during the reaction is insignificant. As a consequence, one can equate the whole process to pseudo-first-order kinetics:

$$\ln\frac{A}{A_0} = -k't \quad (1)$$

According to Eq. (1) a plot of ln[A] versus time (t) would produce a straight line with a slope of k' (the rate constant). The plot at 20 °C of ln h_i versus t was linear ($r^2 > 0.99$) (Fig. 10a). A similar treatment was performed in the temperature range 20–40 °C and a series of kinetic plots was generated. The slopes represent the rate constant of the conversion of acetylenic aldehyde at the respective temperatures (Table 1).

The slope of the lines increased with an increase in temperature. It was also determined that the rate constant was independent of the flow and the slope of the gradient. The results were reproducible from column to column.

The relationship between the rate constant k' and the temperature is given by the Arrhenius equation:

$$\ln k' = -E_a/RT + \ln C \quad (2)$$

where E_a is the activation energy, R is the gas constant, ln C is a constant and T is the temperature (K). According to Eq. (2), a plot of ln k' versus the reciprocal of the absolute temperature defines a straight line of slope $-E_a/R$ and intercept ln A. A straight line was obtained ($r^2 > 0.9$) (Fig. 10b), and the activation energy obtained from the slope of the graph was 12.7 kcal/mol, which corresponds approximately to a doubling of the rate constant with a 10 °C temperature increase [23]. A line extrapolated to the x-axis corresponding to the smallest rate constant led to the temperature where the reaction rate is so small that the chromatographic system would not be able to detect any reaction/fronting and provide resolution between the carbinolamine and the diethylamine Michael adduct. This temperature was determined to be −5 °C. Overlay of chromatograms at −5 °C with the acetylenic aldehyde (analyzed as the carbinolamine), an authentic sample of diethylamine Michael adduct and a sample of acetylenic aldehyde spiked with diethylamine Michael adduct are shown in Fig. 11. No fronting was observed and the carbinolamine peak was found to be spectrally pure, consisting solely of carbinolamine. It was demonstrated that the two species had different affinities for the stationary phase and that resolution of the diethylamine Michael adduct and carbinolamine could be achieved. Fig. 12 shows the separation at −5 °C of an actual acetylenic aldehyde reaction mixture.

Table 1
Influence of temperature on the rate constant of interconversion

T (°C)	k' (min^{-1})	r^2
20	0.69	0.9949
25	1.21	0.9989
30	1.70	0.9827
35	2.33	0.9956
40	2.85	0.9977

4. Conclusion

The in-situ reaction of an acetylenic aldehyde with diethylamine within an HPLC system results in a complex reaction, which can influence the observed chromatography. The reactions can be controlled by utilizing a low column temperature, leading to the formation of a secondary carbinolamine confirmed

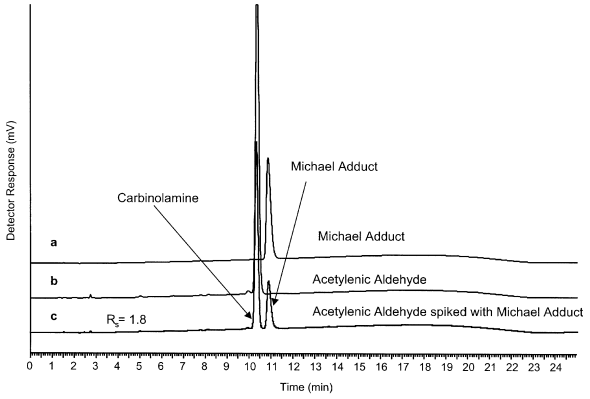


Fig. 11. Separation of diethylamine Michael adduct and acetylenic aldehyde analyzed as carbinol amine at −5 °C. HPLC conditions as in Fig. 3. (a) Acetylenic aldehyde spiked with diethylamine Michael adduct. (b) Acetylenic aldehyde. (c) Michael Adduct.

by LC–MS and NMR experiments. The kinetic analysis along with the Arrhenius plot showed that, at low temperature, the reaction is slow and the system produces only one derivatized species, the secondary carbinolamine. The procedure demonstrated that a rate constant could be obtained from a single chromatographic experiment and the interconversion of the multiple species formed could be controlled by the temperature. At −5 °C the peak was homogenous, corresponding to the carbinolamine. Based on the UV spectra, the maximum absorbance was obtained at 220 nm at −5 °C, allowing for high sensitivity with no column overloading and adequate resolution between all in-process intermediates.

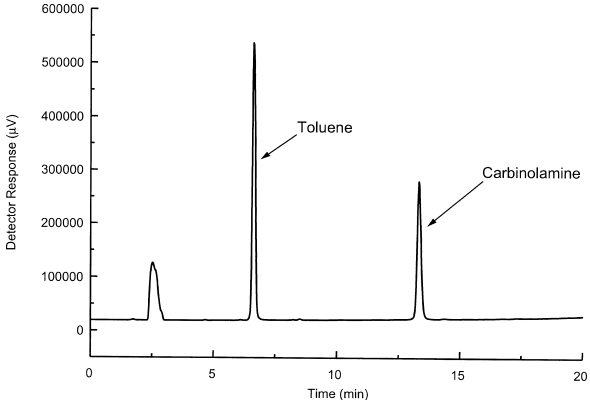


Fig. 12. Toluene concentrate of acetylenic aldehyde.

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