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Trace level detection and quantitation of ethyl diazoacetate by reversed-phase high performance liquid chromatography and UV detection

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Abstract

A method using reversed-phase high performance liquid chromatography (HPLC) with UV detection has been developed and validated for the trace level (ng/mL) detection and quantitation of ethyl diazoacetate (EDA), a toxic impurity, in sample matrix. Method development included the evaluation of several analytical techniques including LC–MS and GC–MS, which in this case, proved to be unacceptable means of analysis. The chromatographic separation employed in this method utilizes a mobile phase system of acetonitrile and water with analysis carried out using UV detection at 250 nm. The final method showed excellent linearity, accuracy, repeatability, specificity and recovery when evaluated at the quantitation limit (QL) of 6 ng/mL.

Keywords: Ethyl diazoacetate; Trace level detection; Cyclopropanation; HPLC; UV detection; Diazo compounds

1. Introduction

Ethyl diazoacetate (EDA) (**I**) (Fig. 1) is often used as a carbenoid or carbene precursor for the cyclopropanation of alkenes [1]. Reactions involving its use can be found in the pharmaceutical industry for the formation of compounds used as synthetic starting materials, intermediates or final active pharmaceutical ingredient (API) [2]. Ethyl diazoacetate is known to be toxic (oral: rat, median lethal dose 400 mg/kg; intravenous: rat, median lethal dose 280 mg/kg) and is thought to be a potential carcinogen/mutagen [3,4]. If this compound were used in or associated with a synthetic route and there was concern that it may not be eliminated during the processing steps of the reaction, its residual content should be investigated at a toxicologically acceptable level. Such trace level determinations may be difficult.

Although the use of EDA in synthetic organic chemistry is well documented [1], trace level detection is not; probably in part due to its reactivity. EDA is part of a class of compounds, which are known to be very reactive. It is known to be heat sensitive, emit toxic fumes and has the potential to explode when heated [3]. Work on EDA, including detonation properties and thermal stability, has been performed to show that it is safe for large scale use in pilot plant facilities [5–8]. Results based on thermal stability data indicate that EDA does not show a proclivity for detonation [5]. Work on EDA decomposition has been carried out using head space gas chromatography (HS-GC) and results indicate that its major decomposition products include carbon dioxide, ethane and nitrogen [6]. Other work has shown that decomposition results primarily in the formation of high boiling esters [6,7]. The onset temperature at which this decomposition occurs can be as low as 104 °C at a heating rate of 1 °C/min as measured by differential scanning calorimetry (DSC) [9]. However, accelerated rate calorimetry (ARC) data suggests an onset decomposition temperature as low as 55 °C (97 wt%) and is proportional to the EDA concentration [6].

This paper describes the development and validation of a reversed-phase liquid chromatographic (LC) method with UV detection for trace level detection and quantitation of EDA, a toxic and potentially carcinogenic/mutagenic carbenoid or carbene precursor for the cyclopropanation of alkenes. The initial approaches investigated for method development are also

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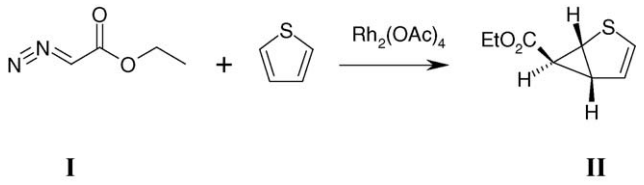

Fig. 1. Cyclopropanation scheme containing the structures of ethyl diazoacetate (**I**) and cyclopropanated alkene (**II**) sample matrix.

described and compared which include gas chromatography-mass spectrometry (GC-MS), GC using cool-on column injection (OCI) and reversed-phase high performance liquid chromatography-mass spectrometry (HPLC-MS) using electrospray ionization (ESI) in both the positive and negative modes.

2. Experimental

2.1. Chemicals

Ethyl diazoacetate (containing ≤10% dichloromethane) and ammonium acetate were purchased from Sigma–Aldrich Chemical Company Incorporated (St. Louis, MO, USA). HPLC grade acetonitrile, water and methanol were purchased from Honeywell Burdick and Jackson (Muskegon, MI, USA). GC grade ethyl acetate was purchased from Mallinckrodt Baker Inc. (Phillipsburg, NJ, USA). A sample of compound **II** (Fig. 1) was obtained from the Chemical Product Research and Development Laboratories at Eli Lilly & Company (Indianapolis, IN, USA).

2.2. Standard stock solution preparation

For HPLC analysis and method validation, a 1 mg/mL stock solution of EDA was prepared in a mixture of water and acetonitrile (85:15, v/v). Linearity standards were prepared by dilution from this single stock solution. Samples for HPLC-MS evaluation were prepared at a concentration of 1.0 mg/mL in acetonitrile and at 10 mg/mL in a mixture of 3 mM NH_4OAc in methanol/water (80:20, v/v). The GC-MS samples were prepared at a concentration of 11 mg/mL in a mixture of water and acetonitrile (60:40, v/v). For OCI, a 2.6 mg/mL stock solution of EDA was prepared in ethyl acetate. Linearity standards were prepared by dilution from this single stock solution.

2.3. Spike and recovery stock solutions and sample preparation

Three concentrations of EDA were prepared as stock solutions (62.0; 247.8; 1239.0 ng/mL). These solutions were then spiked 1:10 into each one of 18 solutions containing the sample matrix (Compound **II**, Fig. 1). A total of six solutions per concentration level were prepared. The sample matrix solutions were prepared at a concentration of 10 mg/mL using solid compound of 99.4% purity (Compound **II**, Fig. 1). Dissolution of this solid material was accomplished with a mixture of acetonitrile and water (60:40, v/v).

Table 1
HPLC conditions for the analysis of EDA

Column	C_{18} 4.6 × 150 mm 3.5 μm
Flow rate (mL/min)	1.5
Wavelength (nm)	250
Column temperature (°C)	25
Injection volume (μL)	10.0
Gradient[a]	
0 min	85% A and 15%B
10 min	30% A and 70% B
11 min	85% A and 15% B
15 min	85% A and 15% B
Total run time (min)	15.0

[a] Mobile phase A: water; mobile phase B: acetonitrile.

2.4. Instrumentation and software

2.4.1. HPLC and HPLC-MS

All liquid chromatographic analysis was conducted on an Agilent 1100 series HPLC equipped with a UV–vis variable wavelength detector set at 250 nm, Agilent Technologies Inc. (Palo Alto, CA, USA). Separations were obtained on a SunFire™ C_{18} column (4.6 mm × 150 mm 3.5 μm), Waters Corporation (Milford, MA, USA). The remaining HPLC conditions are detailed in Table 1. The data were collected via TOTALCHROM™ Version 6.2, PerkinElmer™ Instruments LLC (Shelton, CT, USA). The HPLC-MS analysis was conducted on an Agilent 1100 series HPLC, equipped with a photo diode array detector (PDA), Agilent Technologies Inc. (Palo Alto, CA, USA). The instrument was coupled to a Micromass ZMD, single quadrupole mass spectrometer, operating in positive and negative electrospray ionization mode, Micromass UK Ltd. (Manchester, UK). Separations were obtained on an Xterra® MS C_{18} (2.1 mm × 50 mm 3.5 μm) column, Waters Corporation (Milford, MA, USA). These data were collected with MassLynx Software, Version 3.5, Micromass UK Ltd. (Manchester, UK).

2.4.2. OCI and GC-MS

Analysis utilizing OCI was performed on an Agilent 6890N series GC equipped with a flame ionization detector (FID), Agilent Technologies Inc. (Palo Alto, CA, USA). Separations were obtained on an Agilent DB-1701 (30 m × 0.25 mm i.d. × 0.25 μm film) GC column preceded by an Agilent FS, deactivated (0.53 mm i.d. × 1 m) retention gap, Agilent Technologies Inc. (Palo Alto, CA, USA). The remaining OCI conditions are detailed in Table 2. The data were collected via TOTALCHROM™ Version 6.2, PerkinElmer™ Instruments LLC (Shelton, CT, USA). The GC-MS analysis was conducted on an Agilent series 6890 GC linked to a 5973N MSD mass spectrometer, Agilent Technologies Inc. (Palo Alto, CA, USA). Separations were obtained on an Agilent DB-Wax (30 m × 0.25 mm i.d. × 0.25 μm film) GC column, Agilent Technologies Inc. (Palo Alto, CA, USA). These data were collected via MSD Chemstation Software, also by Agilent Technologies Inc.

2.4.3. UV–vis spectroscopy

UV measurements were determined using an Hewlett-Packard (HP) 8453A UV–vis spectrophotometer, Agilent

Table 2
OCI conditions for the analysis of EDA

Column	DB-1701 (30 m × 0.25 mm i.d. × 0.25 μm film)
Retention gap	FS, deactivated (0.53 mm i.d. × 1 m)
Carrier gas	Helium flow of 1.7 mL/min at 77 °C (17.2 psi) constant flow mode
Injection temperature (°C)	77 (track oven)
Detection temperature (°C)	280 (FID)
Injection volume (μL)	1.0
Oven temperature program	
Initial temperature	77 °C hold for 4.0 min
Ramp 1	15 °C/min to 130 °C
Final temperature	130 °C hold for 8.35 min
Run time (min)	15.0

Technologies Inc. (Palo Alto, CA, USA) equipped with UV–vis ChemStation Rev. A. 08.03 Software also by Agilent Technologies Inc.

3. Results and discussion

3.1. GC-MS evaluation

Based on the thermal data previously cited, analytical techniques requiring heat or other types of energy may not be appropriate for accurate quantitative analysis, especially at trace levels. Several analytical techniques were explored to determine suitability for trace level detection and quantitation of EDA. Thermal decomposition was explored through the use of GC-MS using a split injection at 225 °C with an initial oven temperature of 30 °C. This strategy resulted in an excessive release of CO_2, the formation of diethyl maleate, diethyl fumarate and ethyl orthoformate, all of which are consistent with literature citings [9]. Furthermore, as shown in Fig. 2, several major decomposition products were observed indicating that the decomposition of EDA may involve a complicated series of reactions [8]. Although GC-MS is typically considered to be a useful, sensitive technique for volatile and semi-volatile compounds, significant consideration must be given to compounds that dissociate into low molecular weight fragments. A complicating factor associated with the positive identification of EDA is its fragmentation pattern. Observed fragments of *m/z* 69, *m/z* 41 and *m/z* 29 are in the range of background noise from the atmosphere due to molecules such as nitrogen *m/z* 28 and carbon dioxide *m/z* 44. This can reduce signal to noise ratio and any subsequent detection level (DL). It was therefore concluded that trace level quantitation at these low molecular weights can be difficult and that GC-MS was not the preferred technique.

3.2. Evaluation of OCI

In an attempt to address thermal degradation observed with GC-MS, it was hypothesized that OCI may lower the GC inlet temperature enough to prevent EDA decomposition. A method was developed for OCI using an injection temperature of 77 °C. A linearity assessment at five EDA concentrations was performed using the conditions described in Table 2. A coefficient of determination (r^2) of 0.9999 was observed. To ensure the EDA response factor remained constant at varying temperatures, the effect of inlet temperature on peak response was evaluated. As shown in Fig. 3, response factors for EDA varied significantly at different inlet temperatures, which also resulted in variable recovery values. This observation is consistent with previous ARC data [6]. A challenge with using a 40 °C inlet temperature or lower in OCI, is the re-equilibration time of the GC. In this case, the GC used was not equipped with a cryogenic

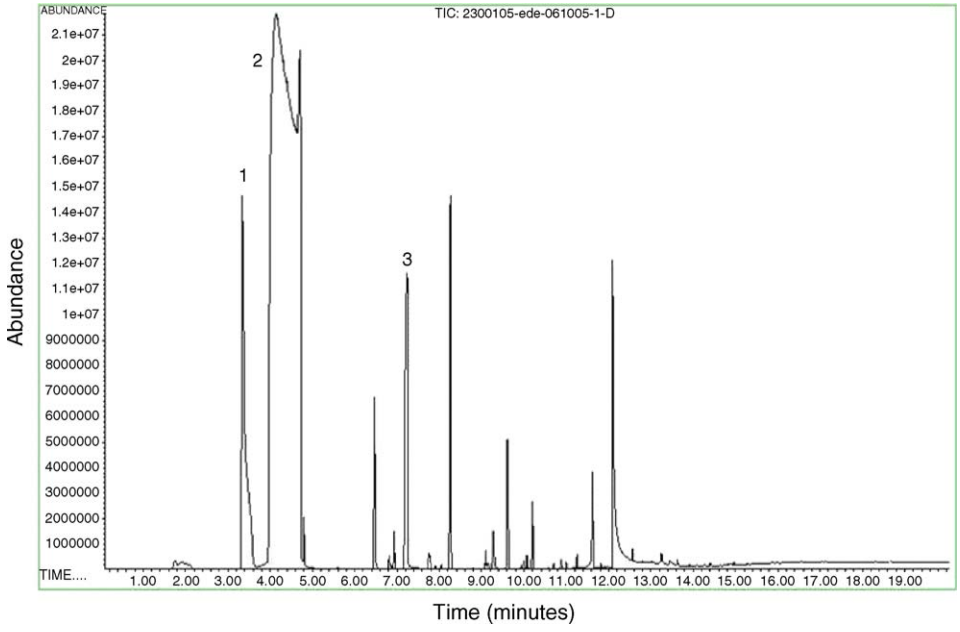

Fig. 2. GC-MS chromatogram of EDA at 11 mg/mL. Peaks 1 and 2 represent solvents, dichloromethane and acetonitrile respectively. Peak 3 represents EDA. All other peaks are decomposition products suggesting the decomposition of EDA may involve a complicated series of reactions.

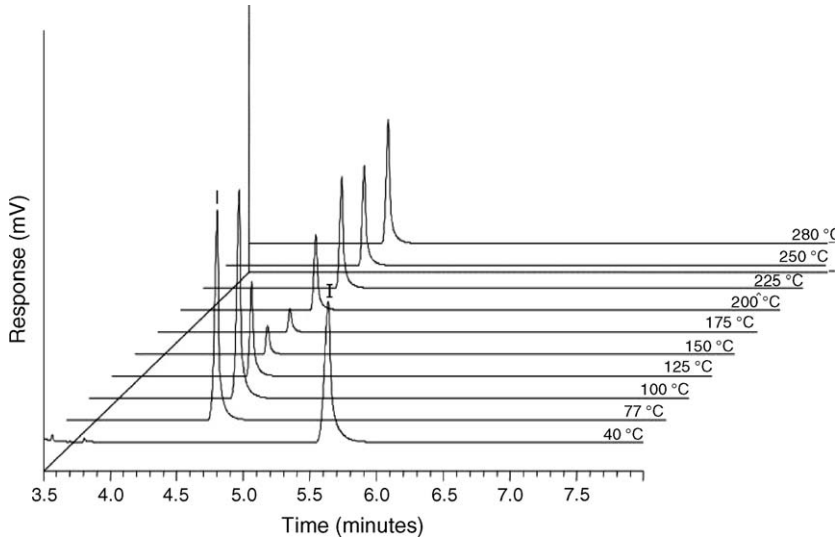

Fig. 3. OCI chromatogram of EDA (**I**) at 0.26 mg/mL. This chromatogram shows the variability of EDA peak response at various injection temperatures. *Note:* The lengthened retention time of the EDA peak at 40 °C is believed to be a function of the volatility of EDA (flash point = 26 °C). Also note, the absence of observed degradants at the upper temperature range (280 °C). It is suspected that the deposition of EDA directly on the GC column may have prevented degradation.

cooling system that would facilitate cooling of the inlet to ambient temperatures or below. Consequently, higher temperatures were evaluated; however, it appeared that the response of EDA decreased on elevation of the inlet temperature from 60 to 175 °C after which the response again increased (Fig. 3). At elevated temperatures it is acknowledged that direct injection to the head of the GC column is not appropriate as it may cause an increase in inlet pressure resulting in material loss. It was hypothesized that the design of the GC instrument may be responsible for this phenomenon and therefore, it will not be discussed further.

Another important factor requiring consideration when performing OCI for trace level detection and quantitation is the amount of sample matrix applied to the GC column (sample loading). In this particular assessment, a 10 mg/mL sample matrix concentration was examined (Fig. 4) to ensure detection sensitivity. This large sample matrix concentration, may be harmful to the GC column and may create baseline noise resulting in potential reduction in sensitivity. Initial OCI data indicated alternate methods should be explored, and if quantitation was attempted using GC-MS or OCI techniques, incorrect results may be obtained.

3.3. HPLC-MS evaluation

HPLC-MS is widely used in the pharmaceutical industry for trace level detection and quantitation of carcinogenic/mutagenic compounds [10]. HPLC-MS typically provides excellent detection of polar and weakly non-polar, ionic, neutral and high molecular mass compounds. The most common ionization approach for HPLC-MS is electrospray ionization (ESI) [10]. Due to the limited fragmentation inherent to the ESI ionization process, it is well known to be the 'softest' of all mass

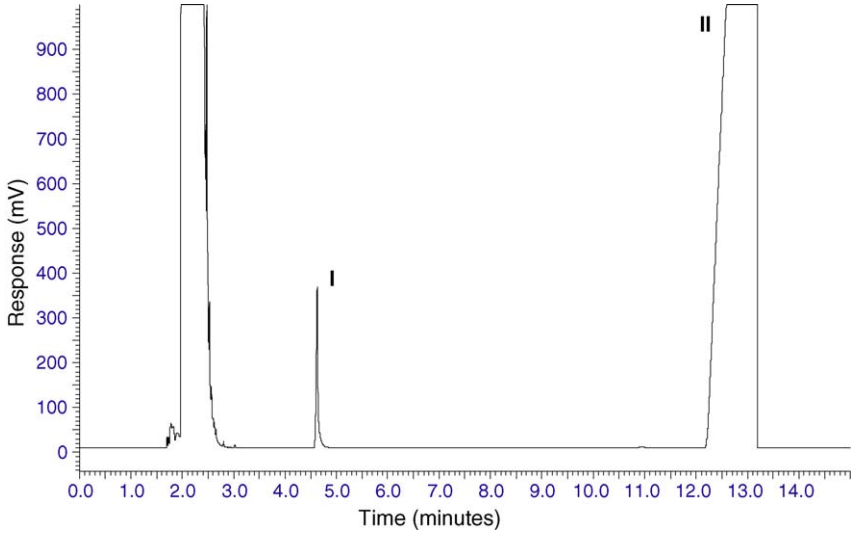

Fig. 4. OCI chromatogram of EDA (**I**) in the presence of cyclopropanated alkene (**II**) sample matrix. A 10 mg/mL sample matrix concentration is shown. This large sample concentration can be detrimental to the GC column as well as creating baseline noise resulting in potential reduction in sensitivity.

Table 3
Validation data obtained for EDA using HPLC with UV detection

Validation criterion	Experiment		Result
Specificity	EDA separated from impurities contained in the sample matrix of interest. EDA also separated from two dimers		All impurities were separated from EDA as observed in Fig. 6
Linearity			
EDA (out of matrix) ($n=6$)	5.0–100.0 ng/mL (0.01–0.20% of nominal sample matrix concentration, 0.05 mg/mL)		$R^2=0.99997$; $Y=5.0\times10^4X+7$
	12.0–75.0 μg/mL (24–150% of nominal sample matrix concentration (0.05 mg/mL)		$R^2=0.99996$; $Y=4.9\times10^7X+3638$
Accuracy and repeatability	Concentration (ng/mL)	Recovery ($n=6$, %)	R.S.D. ($n=6$, %)
EDA (in-matrix)	6.2	104	13.8
	24.8	96	1.3
	124.0	105	0.7
Repeatability – nominal	Concentration (μg/mL)	R.S.D. ($n=5$, %)	
EDA	62	0.4	
Quantitation limit (QL)	Calculated as outlined in ICH Q2B, based on the measured signal-to-noise ratio (10:1) samples with known low concentrations		6 ng/mL
Detection limit (DL)	Calculated as outlined in ICH Q2B, based on the measured signal-to-noise ratio (3:1) samples with known low concentrations		1.0 ng/mL

spectrometric ionization techniques [11]. Unlike electron impact (EI) ionization utilized in GC-MS, ESI does not cause collision-induced disassociation (CID) of the target analyte. Furthermore, ESI can be used in either positive or negative ion-mode or both simultaneously, allowing for detection of protonated and deprotonated forms of the molecule of interest. Compounds typically analyzed using ESI include polar, high molecular mass and ionic molecules.

Initially, 1.0 and 10 mg/mL samples of EDA (Mw = 114.1) were evaluated using HPLC-MS, but data obtained from this evaluation were inconclusive since no definitive molecular ion could be ascertained. Although the HPLC-MS source parameters could be evaluated further to reduce the observed fragmentation, it was not pursued in this study given the complex mass spectrum. Furthermore, during the experiment it was observed that EDA had a significant response on the photo diode array detector (PDA).

Although a method could be developed to monitor a particular fragment of EDA using selective ion monitoring (SIM) to overcome some of the challenges encountered in ESI, at a scan range <100 amu and without consistent fragmentation, it may be difficult to develop a sufficiently robust set of conditions.

3.4. UV–vis spectroscopy

To explore possibilities of a simplified approach for detection and quantitation of EDA, UV–vis spectroscopy was used to obtain its molar absorptivity.

Diazo compounds are well known to provide adequate absorption of UV light due to their high degree of conjugation.

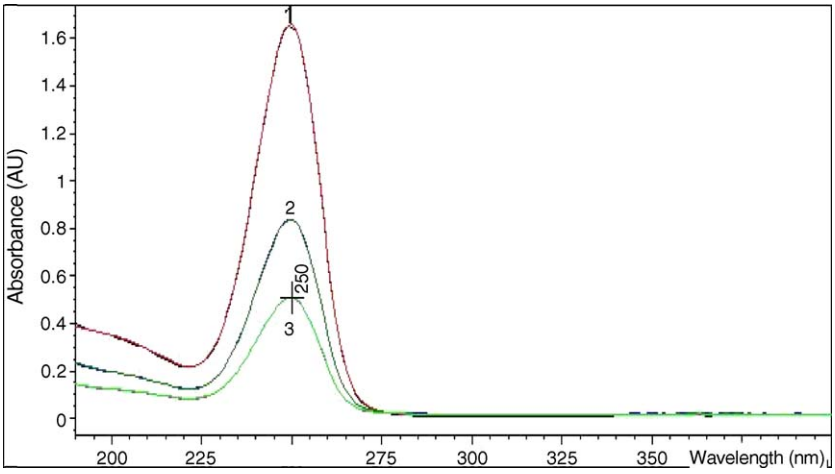

Fig. 5. UV–vis spectra of EDA prepared at: (1) 12.2 μg/mL, (2) 6.1 μg/mL and (3) 3.7 μg/mL in a mixture of water and acetonitrile (85:15, v/v).

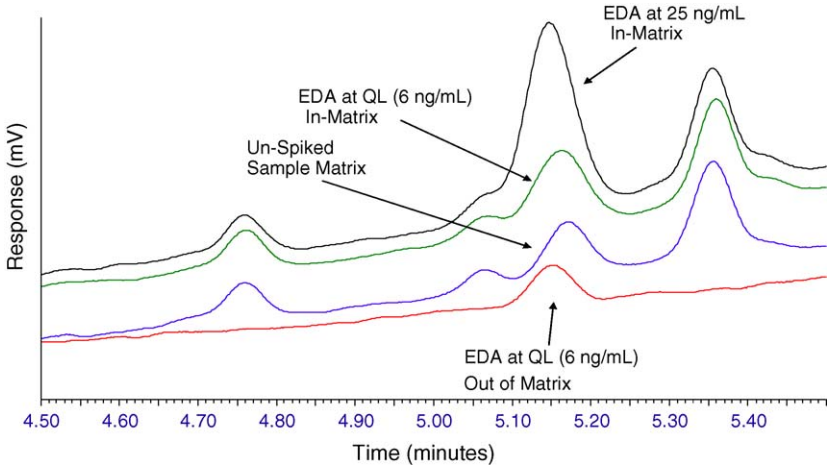

Fig. 6. HPLC chromatogram of EDA (**I**) at the QL, both in and out of the sample matrix (**II**). An un-spiked sample matrix and the 25 ng/mL level are also included for comparison. This chromatogram shows specificity for EDA and that there are no interferences from impurities of the analyte. The challenging sample matrix contained multiple components including EDA near the QL level. This is suspected to be the basis for the increased variability in the recovery at the QL. *Note:* In a separate run PDA data confirmed the identity of EDA.

The molar absorptivity ($\varepsilon_{250\,nm}$) of EDA was measured in triplicate, at three different concentrations, using a UV–vis spectrophotometer (Fig. 5). The average was determined to be 1.56×10^4 L/mol cm. This result indicated that HPLC utilizing UV detection may be a suitable approach for trace level analysis.

3.5. Validation of EDA method by HPLC with UV detection

In the present work, the HPLC conditions listed in Table 1 have been developed and validated for trace level detection and quantitation of EDA. In order to ensure the compound remained stable, HPLC analysis was conducted with a column temperature of 25 °C and in the absence of any buffer component, as it is known that EDA decomposes in the presence of acid [12].

The method showed excellent linearity, accuracy, repeatability, specificity and recovery when evaluated at the QL of 6 ng/mL (Table 3). Furthermore, this method is also selective for two common dimeric reaction products associated with EDA, diethyl maleate and diethyl fumarate.

Linearity was determined using a six point calibration curve from 0.01% to 150% of the nominal concentration 0.05 mg/mL (Table 3). Recovery of EDA from the sample matrix was assessed at three different concentrations using six solutions per concentration ($n = 6$). Results of the spike and recovery studies performed are shown in Table 3. The USP tailing factors of the three concentrations were assessed for each injection. The values range from 0.98 to 1.0, with an average ($n = 18$) of 1.0. A chromatogram of EDA in solution at the QL of 6 ng/mL and when spiked in the sample matrix at the same concentration is shown in Fig. 6. The spike concentration corresponds to 600 ppb of EDA in a sample matrix of 10 mg/mL.

4. Conclusion

Several analytical techniques have been evaluated for trace level detection and quantitation of EDA. Due to the physical properties of this molecule, accurate analysis is challenging and requires a careful approach. Techniques requiring heat or other types of energy may not be appropriate for quantitative assays, especially at trace levels and as shown can cause EDA decomposition.

To overcome inherent molecular instability, an HPLC method has been developed and fully validated for trace level detection and quantitation of ethyl diazoacetate using only UV detection. This method is rapid, accurate and easy to use. It showed excellent linearity, accuracy, repeatability, specificity and recovery when evaluated at the QL. The ability to rely solely on UV detection makes this method highly desirable for fast material screening applications.

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