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# Simultaneous solid phase extraction and derivatization of aliphatic primary amines prior to separation and UV-absorbance detection

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ABSTRACT

To overcome the problem of poor sensitivity of capillary electrophoresis-UV absorbance for the detection of aliphatic amines, a solid phase extraction and derivatization scheme was developed. This work demonstrates successful coupling of amines to a chromophore immobilized on a solid phase and subsequent cleavage and analysis. Although the analysis of many types of amines is relevant for myriad applications, this paper focuses on the derivatization and separation of amines with environmental relevance. This work aims to provide the foundations for future developments of an integrated sample preparation microreactor capable of performing simultaneous derivatization, preconcentration, and sample cleanup for sensitive analysis of primary amines.



## 1. Introduction

Capillary electrophoresis (CE) is an attractive separation technique due to low sample and reagent requirements, speed of analysis, versatility of separation modes, and high separation efficiency. Due to simplicity of instrumentation, on-capillary UV absorbance is the most common detection method integrated to commercial CE instruments. As the result of a short optical pathlength and low injection volume capacity [1], one major drawback of CE-UV is its poor sensitivity. This problem is exacerbated when the target analytes have low or no absorptivity [2]. Among other examples where improvements in sensitivity could allow a wider application of CE-UV [3] and avoid the use of complex and bulky instrumentation [4,5], biogenic amines [6], environmental pollutants [7], and amino acids [8] are of critical importance. The analysis of primary amines such as putrescine, cadaverine, spermidine, and spermine in food samples is clinically relevant because they can cause a variety of health problems including headaches, nausea, and (in some individuals taking monoamine oxidase inhibitors) severe effects on the cardiovascular and central nervous systems [9,10]. Moreover, other amines such as aniline, *p*-anisidine, or aliphatic amines (such as methylamine, propylamine, and pentylamine) can be released to the environment from manufacturing plants linked to the production of dyes, pesticides, and pharmaceuticals [11–14] and affect the surrounding biota (toxicity, bioaccumulation, or generation of secondary compounds with carcinogenic activity). To improve the sensitivity of the analysis of aliphatic amines, derivatization with a chromophore or fluorophore is typically required [3,6]. Although derivatization procedures can produce significant advantages in terms of sensitivity, they also increase the sample demands, preparation time, and costs.

In order to address the necessity of derivatization and other sample preparation requirements of amines in real samples, this paper describes a widely applicable, covalent immobilization approach to extract and derivatize amines prior to their analysis by CE-UV. As described, the approach is based on the use of a chromophore, attached to the surface of thiolated silica particles, which is activated to covalently bind to primary amines. Since the solid phase only retains the analytes of interest, the derivatization also acts as sample cleanup, as extraneous salts and interferences in the sample matrix can be excluded from analysis. After the extraction and derivatization steps are complete a disulfide bond can be cleaved, releasing the tagged amines and allowing the facile regeneration of the surface of the beads. Thus, this solid phase sample preparation has several relevant advantages over traditional derivatization techniques including the ability of integration

*Abbreviations:* –SH particles, 3-mercaptopropyl-functionalized silica gel; CE, capillary electrophoresis; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; NHS, *N*-hydroxysuccinimide; MES, 2-(*N*-morpholino)ethanesulfonic acid hemisodium salt; NTB, 2-nitro-5-thiobenzoic acid; SDS, sodium dodecyl sulfate; TCEP, *tris*(2-carboxyethyl)phosphine.

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for on-line sample preparation [15,16], reducing sample preparation time and cost.

## 2. Experimental section

### 2.1. Materials

All chemicals were analytical reagent grade and used as received. The 3-mercaptopropyl-functionalized silica gel (–SH particles, 200–400 mesh), thiopropyl-sepharose 6B, 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC), *N*-hydroxysuccinimide (NHS), sodium dodecyl sulfate (SDS), tryptamine, methylamine, propylamine, pentylamine, aniline, and *p*-anisidine were obtained from Sigma-Aldrich (St. Louis, MO). Dimercaptotriazine-loaded silica was purchased from Silicycle (Quebec City, Canada). Isopropanol and methanol were obtained from Fisher Scientific (Pittsburg, PA) and JT Baker (Center Valley, PA), respectively. *Tris*(2-carboxyethyl)phosphine (TCEP) was obtained from Biosynth (Switzerland). Solutions of sodium phosphate monobasic (Fisher Scientific, Pittsburg, PA) and 2-(*N*-morpholino)ethanesulfonic acid hemisodium salt (MES) and sodium tetraborate decahydrate buffers, both from Sigma-Aldrich (St. Louis, MO), were adjusted with 1.0 M hydrochloric acid (EMD Chemicals, Philadelphia, PA) or 1.0 M sodium hydroxide (Fisher Scientific, Pittsburg, PA) to obtain buffer with desired pH. The pH measurements were performed with a glass electrode and a digital pH meter (Orion 420A+, Thermo, Waltham, MA). All aqueous solutions were prepared using 18 MΩ cm water (NANOpure Diamond, Barnstead, Dubuque, IA). Deuterium oxide was obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA). Soil samples were collected from the Atacama Desert, Chile (S24°03.629; W69°52.092) $<1$ cm deep from the exposed duracrust.

### 2.2. Instrumentation

A Beckman–Coulter P/ACE MDQ (Fullerton, CA) CE system and fused silica capillaries (50 μm i.d. × 360 μm o.d. × 45 cm length; Polymicro Technologies, Phoenix, AZ) were used. Data acquisition was performed using Karat 32 software (Beckman–Coulter; Fullerton, CA) on an IBM personal computer. Samples were introduced into the capillary by a 3.0 s, 0.2 psi pressure injection, and subsequently separated by an applied potential of 25 kV at 25 °C. Direct UV detection was performed using a wavelength of 200 nm, through the capillary at a window located 35 cm from the inlet. (The proposed derivatization scheme introduces a chromophore to the analyte with an absorption maximum at 412 nm, however, due to poor signal/noise of our instrument at the available 400 nm filter, a lower wavelength was selected for the analyses.) The separation buffer consisted of 10 mM borate, 50 mM SDS and 20% (v/v) methanol. All off-line experiments involving UV–vis absorption were performed using a GENESYS10 spectrophotometer (Thermo Electron, Waltham, MA). The products of the derivatization procedure were characterized by nuclear magnetic resonance ($^1$H-NMR) in $D_2O$ using a Varian INOVA 500 MHz Spectrometer at 25.0 °C with a relaxation delay of 2.00 s, a pulse of 24.5°, acquisition time of 2.500 s, width of 7995.2 Hz, and 16 repetitions.

### 2.3. Derivatization procedure

The derivatization scheme for primary amines is summarized in Fig. 1. First, 0.02 g of –SH particles (1.2 mmol $g^{-1}$ loading of –SH groups) were reacted with 1.0 mL of 60 mM DTNB in 100 mM phosphate buffer at pH 8.0 for 10 min [17,18]. A disulfide exchange occurs, covalently linking half of the DTNB molecule, 2-nitro-5-thiobenzoic acid (NTB), to a thiol group on the particle, leaving an equivalent of NTB in solution [19]. After rinsing the excess NTB from the particles, the carboxylic group of the immobilized NTB tag was activated with EDAC to form a highly reactive *o*-acylisourea intermediate [19]. Because EDAC can easily hydrolyze, it was subsequently replaced by NHS, yielding a semi-stable NHS ester that is still highly reactive toward amines [19]. This activation was achieved by shaking the particles in 0.50 mL of 100 mM EDAC and 100 mM NHS in isopropanol (rather than in aqueous solution in order to inhibit hydrolysis of the activated carboxylic group [20]) for 30 min. Next, the immobilized, activated NTB tag was allowed to react under gentle agitation for 1 h with the selected amines contained in 1.0 mL of 50 mM MES buffer at pH 6.0. This

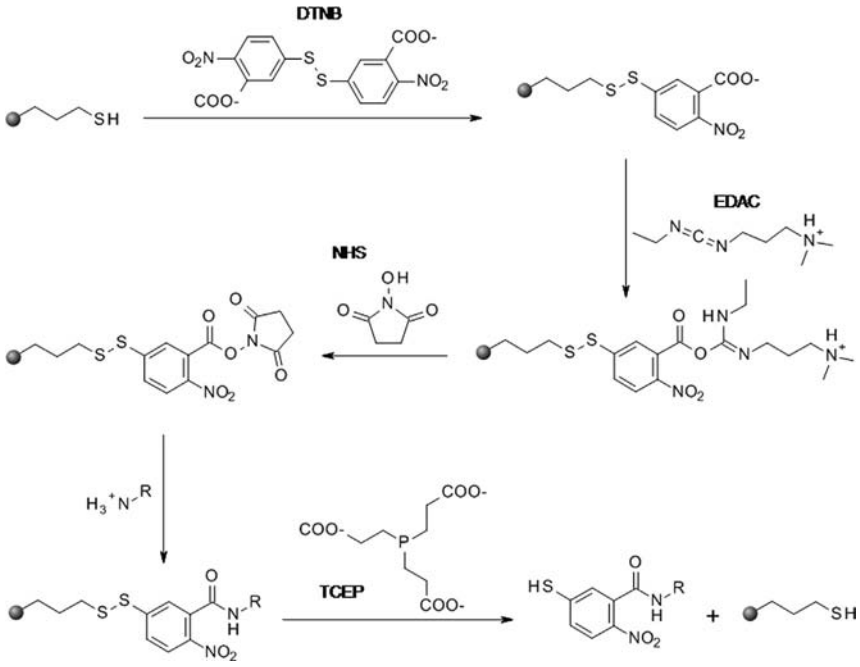


**Fig. 1.** Scheme for solid phase derivatization of primary amines. See Section 2.3 for details.

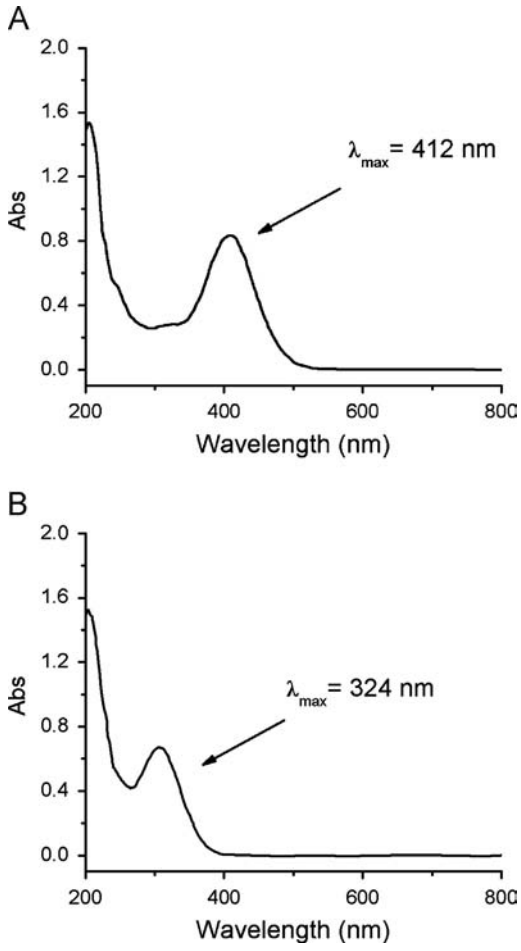


**Fig. 2.** Spectral curves of flow-through after exposing (A) –SH particles and (B) silica particles to a solution of DTNB in limiting reagent quantities.

step resulted in the formation of an amide bond, linking the NTB and the amines [19]. After copious rinsing of the particles, 1.0 mL of 30 mM TCEP (an odorless, water-soluble, and pH-stable disulfide reducing agent [21]) dissolved in the separation buffer was mixed with the particles for 10 min to reduce the disulfide bond, releasing the tagged amines from the particles for subsequent separation and detection, and leaving regenerated –SH particles. All reaction times were optimized to achieve maximum yields with the least required time. For the real sample analysis, 15 mg of soil was extracted by sonication with 1.5 mL of 50 mM MES buffer at pH 6.0 for 10 min. Spiked samples included 10 mM each of pentylamine, propylamine, methylamine, aniline, and *p*-anisidine in the 1.5 mL of MES buffer containing the soil. After centrifugation, 1.0 mL of supernatant was added to the activated particles and analyzed according to the procedure described above.

## 3. Results and discussion

### 3.1. Attachment of NTB to –SH particles

DTNB is a convenient chromophoric tag to use because it has a pale yellow color in solution ($\lambda_{max}$=324 nm) that shifts to orange ($\lambda_{max}$=412 nm) when split into NTB. This color change allows confirmation of attachment of the tag (NTB) to the –SH particle. Fig. 2 shows the spectral curves obtained by an external spectrophotometer of the washes performed on –SH particles (A) and plain silica particles (control, B). Initial washes of both types of particles did not render relevant signals in the UV–vis spectra. As can be observed in Fig. 2A, after mixing the –SH particles with a solution containing DTNB (in limiting reagent quantities), the flow-through had an absorbance maximum at 412 nm, indicating the presence of NTB (and therefore confirming a disulfide exchange). (As a side note, DTNB was used in limiting reagent quantities in this experiment in order to obtain a flow-through containing mainly one species, NTB, yielding a simple spectral curve with one major identifying feature. When DTNB was used in excess of the number of thiol groups, overlapping features of DTNB and NTB were both present in the spectrum; i.e., there was a peak at both 324 nm and 412 nm (data not shown). Subsequent experiments involving the optimization and quantification of the attachment of the chromophore to the particles used excess quantities of DTNB to maximize the amount attached.) On the other hand, when performing the same experiment with the plain silica particles (control), the flow-through had an absorbance peak at 324 nm, confirming the presence of unreacted DTNB (Fig. 2B). No absorbing species were present in the final washes performed to discard nonspecifically bound reagent from each type of particle. As mentioned before, TCEP cleaves the disulfide bond formed between the –SH particle and NTB. When the NTB-modified –SH particles of Fig. 2A were mixed with TCEP, a spectral curve of the flow-through gave an absorbance peak at 412 nm, indicating the release of the NTB tag (data not shown). On the other hand, when the silica particles of Fig. 2B were mixed with TCEP, a featureless spectrum was obtained, indicating no release of covalently bound or nonspecifically adsorbed NTB (data not shown).

Then, to quantify the yield of this step, the attachment efficiency of the NTB tags to the –SH particles was investigated. For these experiments, the –SH particles were allowed to react with an excess of DTNB. After washing the particles to remove any adsorbed DTNB or NTB, the bound tags were released by reduction with TCEP and analyzed by CE-UV absorbance based on a calibration curve of NTB in solution (data not shown). According to the results, an NTB attachment efficiency of 25 $\pm$ 2% to the –SH groups available on the particles (1.2 mmol $g^{-1}$, as reported by the manufacturer) was achieved. Further attempts to optimize this step and to boost the attachment efficiency by either using more polar protic solvents [22–24] or by initially washing the –SH particles with TCEP (to reduce any oxidized sulfhydryl groups) [23] did not increase the yield. Alternative solid supports including thiopropyl-sepharose 6B (Sigma-Aldrich) and dimercaptotriazine-loaded silica (Silicycle) beads of the same size did not yield better results.

Subsequent experiments aimed at optimizing the cleaving step showed that all of the tags were released with the first exposure to TCEP (data not shown). Additionally, the selected –SH particles proved to be regenerable, as reloading and then recleaving of the NTB tags yielded the same attachment efficiency as that obtained during the first cycle (25 $\pm$ 2%). It is important to note that after the attachment of NTB to the –SH particles, exposure to basic pH levels during subsequent rinsing steps or during activation and attachment of amines should be avoided. This exposure may cause premature alkaline disruption of the disulfide bond [25]. Thus, MES at pH 6.0 was considered a suitable buffer for attachment. The pH of the TCEP cleaving solution must also be kept low in order to avoid auto-oxidation of the thiols into disulfides [18]. Therefore, dissolving the TCEP in the separation running buffer was suitable for retaining stability of derivatized products.

### 3.2. Activation of NTB tags and attachment of amines

Once the NTB tags were attached to the –SH particles, the carboxylic groups of NTB were activated with EDAC and NHS and then reacted with primary amines (as shown in Fig. 1). After rinsing, the amines, attached to the NTB tags, were cleaved off of

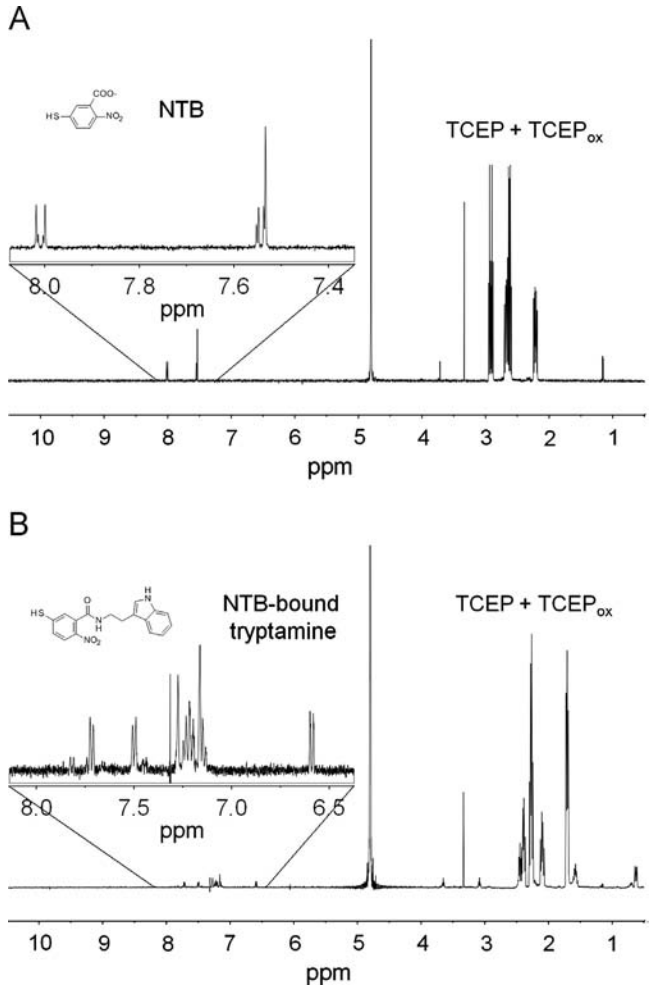


**Fig. 3.** $^1$H-NMR spectra of (A) NTB tags cleaved off of –SH particles by TCEP in $D_2O$ and (B) NTB tags with bound tryptamine cleaved off of –SH particles by TCEP in $D_2O$. $^1$H-NMR conditions as described in Section 2.

the particles using TCEP and analyzed by NMR spectroscopy. Fig. 3A shows a control experiment in which a solution containing the released NTB tag, with no amine attached, was investigated. As can be observed, the signals corresponding to the aromatic protons of the NTB molecule are present in the downfield region of the spectrum [26] (highlighted in the inset), again indicating successful attachment and cleavage of the chromophoric tag. Fig. 3B shows that when the immobilized tags are exposed to EDAC and NHS for activation and then to a primary amine (tryptamine, in the case shown in the figure), and then cleaved with TCEP, a significantly different spectrum was obtained. In addition to the NTB aromatic proton signals, the corresponding downfield-shifted aromatic proton signals of tryptamine [26] were present as well (all highlighted in the inset). Also present in both spectra of Fig. 3 is a reference solvent signal of deuterium oxide at 4.811 ppm and signals of TCEP and oxidized TCEP in the upfield region, below 4 ppm. Thus, these experiments confirmed the successful coupling of amines to the activated tags. Again, control experiments were performed to confirm the absence of nonspecific binding/adsorption of amines to plain silica particles.

### 3.3. Signal enhancement and calibration curves

In order to critically evaluate the performance of the proposed methodology, calibration curves were performed with five model amines using CE-UV absorbance. Pentylamine, propylamine, and

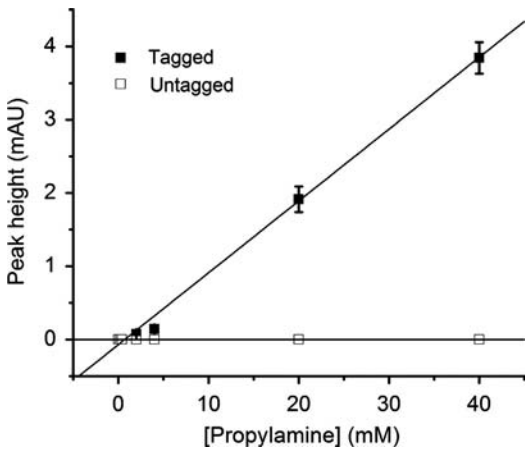


**Fig. 4.** Calibration curve of untagged and tagged propylamine. CE conditions: 50 μm i.d. × 45 cm long fused silica, 3.0 s, 0.2 psi pressure injection, $V_{sep}$=25.0 kV, 25 °C, 10 mM borate, 50 mM SDS, 20% (v/v) methanol running buffer.

methylamine were selected because they lack native UV absorptivity and the aniline and *p*-anisidine were chosen because they have a clear, well-characterized absorption band in the UV region (product of the $\pi \rightarrow \pi^*$ transitions of the aromatic rings). As shown in Fig. 4, the derivatization method enabled the analysis of propylamine by CE-UV with a sensitivity of $0.095 \pm 0.002$ mAU mM$^{-1}$.

Although this result demonstrates a significant advantage for the detection of non-optically active amines, the derivatization technique significantly affected the response of other compounds that were already chromophoric. For example, in the case of aniline, the sensitivity obtained for the untagged aniline ($2.7 \pm 0.3$ mAU mM$^{-1}$) was significantly larger than the sensitivity obtained upon tagging ($0.072 \pm 0.004$ mAU mM$^{-1}$). Attempts were made to optimize the extent of EDAC/NHS coupling of amines to the chromophore, however, these figures shed light on the inefficiency of the coupling. Work is currently underway to explain this mechanism and design additional tags that can be applied for the derivatization of other (chromophoric) amines. Despite non-ideal coupling efficiency, the derivatization method still improves the sensitivity in detection of aliphatic amines. Without the derivatization, the aliphatic amines would not be detectable by CE-UV. The sensitivity, $R^2$ value of the linear fit, calculated limits of detection (peak height signal-to-noise ratio of 3), and limits of quantitation of all five selected amines analyzed by CE-UV are listed in Table 1. These values were calculated from calibration curves determined for each amine individually. In the case where a mixture of amines in a real sample will be analyzed, it would be important to determine the calibration curves of each amine in the presence of other amines competing for the derivatization. Chemometric methods would be valuable for developing multivariate calibration curves in cases of mixtures of multiple analytes.

### 3.4. Analysis of amines in a soil sample

In order to demonstrate the capabilities of the proposed strategy, a soil sample was spiked (see Experimental Section) with a mixture of amines with environmental relevance: pentylamine, propylamine, methylamine, aniline, and *p*-anisidine. After aqueous extraction (see Experimental Section), the components of the sample underwent the derivatization scheme and then were separated by CE. Fig. 5 shows an electropherogram of the derivatized extract, where all the spiked components can be identified (marked peaks). A few system peaks were also obtained, due to multiple components comprising the separation buffer [27]. The unspiked extraction of soil showed undetectable amounts of the selected amines. This data is comparable with results obtained by

**Table 1**
Analytical figures of merit corresponding to the selected amines.

| Amine | Sensitivity (mAU $mM^{-1}$) | $R^2$ of linear fit (0–40 mM amine) | LOD (mg $L^{-1}$) | LOQ (mg $L^{-1}$) | $t_m$ | $N$ | Resolution (between $peak_n$ and $peak_{n+1}$) |
|---|---|---|---|---|---|---|---|
| Pentylamine | $0.138 \pm 0.007$ | 0.984 | $20 \pm 9$ | 35 | $5.16 \pm 0.06$ | 17000 | 1.2 |
| Propylamine | $0.095 \pm 0.002$ | 0.995 | $16 \pm 4$ | 120 | $5.36 \pm 0.06$ | 14000 | 3.5 |
| Methylamine | $0.036 \pm 0.003$ | 0.948 | $38 \pm 7$ | 62 | $5.87 \pm 0.06$ | 47000 | 6.6 |
| Aniline | $0.072 \pm 0.004$ | 0.980 | $140 \pm 50$ | 190 | $6.77 \pm 0.05$ | 28000 | 1.1 |
| *p*-Anisidine | $0.065 \pm 0.006$ | 0.948 | $90 \pm 10$ | 49 | $6.97 \pm 0.06$ | 16000 | – |

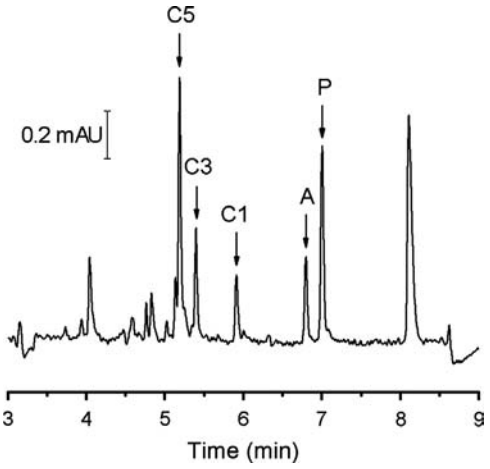


**Fig. 5.** Separation of environmentally relevant amines (C5=pentylamine, C3=propylamine, C1=methylamine, A=aniline, and P=*p*-anisidine) from a spiked (see Section 2) soil matrix after extraction and solid phase sample preparation using the scheme described in Fig. 1. CE conditions: 50 μm i.d. × 45 cm long fused silica, 3.0 s, 0.2 psi pressure injection, $V_{sep}$=25.0 kV, 25 °C, 10 mM borate, 50 mM SDS, 20% (v/v) methanol running buffer.

Skelley et al. who analyzed exposed duracrust samples from same location [28]. The only amines the group found in the sample were very low levels of ethylamine and methylamine. Other amine signals were below blank levels. In contrast to our extraction by sonication at room temperature, the group used a subcritical water extractor, which uses high temperature and pressure to improve extraction efficiency, and LIF detection.

This electropherogram demonstrates successful derivatization, separation, and UV detection of the non-absorbing amines (methylamine, propylamine, and pentylamine), despite having similar structures and molecular weights. Additionally, the procedure also enabled the separation of aniline and *p*-anisidine, which could not be separated under the same separation conditions without derivatization. This experiment shows proof-of-concept of solid phase sample preparation (derivatization and sample cleanup) and analysis of environmentally important amines in a relevant sample matrix. Furthermore, the migration time of each amine (Table 1) was stable ( < 1.2%RSD) over a course of 8 separation repetitions. The plate numbers and resolution between adjacent pairs of peaks are listed in Table 1.

## 4. Conclusions

A novel sample preparation procedure for derivatization has been designed to increase the absorptivity of aliphatic primary amines, enabling CE-UV analysis. This paper has demonstrated successful covalent immobilization of a chromophoric tag to a solid phase, activation and coupling of the tag to primary amines, and finally, cleavage of the tagged amine from the solid phase. The derivatization and subsequent separation of five environmentally relevant amines, three of which are aliphatic, was demonstrated with a soil sample as a real sample matrix. As the derivatization scheme applies to any primary amine, this chemistry has impactful applications in a wide range of areas such as in food, biomedical, environmental, and pharmaceutical sciences. A unique advantage of the solid phase chemistry is that the particles can be regenerated and reused. The particles can be incorporated into commercial capillary cartridges to exploit the automated, high-throughput capabilities of benchtop systems, and also into portable lab-on-a-chip devices for point-of-care testing with minimal sample preparation required of the analyst [29–31]. Preliminary designs of integrated reactors for the derivatization chemistry described include fabrication of frit-free, packed coupled capillaries in the style of Saavedra et al. [32] and also the packing of particles into a LabSmith CapTite™ Union Interconnect. Future work includes improving the extent of coupling (tag to particle and amine to tag) which will allow simultaneous preconcentration to be achieved in addition to the derivatization and sample cleanup, further improving the sensitivity of analysis of amines by CE-UV and expanding the utility and applications of the sample preparation technique.

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