Research Article

N-(4-aminophenyl)piperidine Derivatization to Improve Organic Acid Detection with Supercritical Fluid Chromatography-Mass Spectrometry

Yih Ling Saw[a], John Boughton[a], Faith Wroniuk[a], Mahmoud Elhusse Mostafa[b],
Michael Armbruster[b], Peter J. Pellegrinelli[a], Samantha Calvez[a], Alexander Kaplitz[a], Lark Perez[a],
James L. Edwards[b,*], James P. Grinias[a,*]
[a]Department of Chemistry & Biochemistry, Rowan University, Glassboro, NJ USA
[b]Department of Chemistry, Saint Louis University, St. Louis, MO USA
*Corresponding Authors: J.L.E. (jim.edwards@slu.edu), J.P.G. (grinias@rowan.edu)

Keywords: Supercritical Fluid Chromatography, SFC-MS, Chemical Derivatization, Organic Acids

Abstract

The analysis of organic acids in complex mixtures by LC-MS can often prove challenging, especially due to the poor sensitivity of negative ionization mode required for detection of these compounds in their native form. These compounds have also been difficult to measure using SFC-MS, a technique of growing importance for metabolomic analysis, with similar limitations based on negative ionization. In this report, the use of a high proton affinity N-(4-aminophenyl)piperidine derivatization tag is explored for the improvement of organic acid detection by SFC-MS. Four organic acids (lactic, succinic, malic, and citric acids) with varying numbers of carboxylate groups were derivatized with N-(4-aminophenyl)piperidine to achieve detection limits down to 0.5 ppb. The effect of the derivatization group on sensitivity and mass spectrometric detection are also described. Preliminary investigations into the separation of these derivatized compounds identified multiple stationary phases that could be used for complete separation of all four compounds by SFC. This derivatization technique provides an improved approach for the analysis of organic acids by SFC-MS, especially for those that are undetectable in their native form.

1. Introduction

Monitoring small molecule metabolites can play an important role in understanding complex biological systems and has been used extensively in the areas of clinical diagnostics, drug development, toxicology, and pharmacology [1]. One important class of metabolites is organic acids, which are involved in various metabolic pathways, including the Cori and Krebs cycles [2]. Because of their involvement in these processes, the analysis of organic acids can provide crucial insight into a variety of important cellular mechanisms. However, there are a number of challenges to effectively characterizing these compounds using LC-MS. Because of their high polarity, native organic acids have been separated using HILIC [3], but this approach can require lengthy column re-equilibration steps between injections [4]. Ion-exchange chromatography has also been used for the separation of organic acids, although challenges exist when coupling this technique to MS [5]. Because of their carboxylic acid functional groups, these compounds are typically detected by ESI-MS in negative mode, which generally suffers from lower signal intensity than positive mode [6,7]. These challenges provide motivation for the development of new analytical techniques for the analysis of organic acids.

Supercritical fluid chromatography (SFC) coupled to MS is increasingly being adopted for metabolomic applications because of its compatibility with a broad range of both hydrophilic and hydrophobic compounds [1,8]. Additionally, its faster re-equilibration time provides for higher analytical throughput than HILIC [9]. SFC-MS has specifically been used for the analysis of organic acids in multiple reports [8–10]. However, negative ionization mode is still typically employed for ESI in these methods, which can decrease sensitivity [11]. To resolve this issue, chemical derivatization can be employed to enhance the proton affinity of organic acid metabolites and permit detection in positive mode ESI [12]. Recently, the use of N-(4-aminophenyl)piperidine to derivatize carboxyl groups in organic acids increased sensitivity in LC-MS analysis up to 20-fold relative to the native compound [13]. In this study, this approach was adapted for use in SFC-MS, with a focus on the identification of method parameters that best improved analyte signal. Additionally, multiple stationary phases were tested to explore which provided the best separation of these derivatized compounds.

2. Materials and Methods

2.1. Reagents

L-lactic acid, succinic acid, D-malic acid, citric acid, hexafluorophosphate azabenzotriazole tetramethyl uronium (HATU), dimethyl sulfoxide (DMSO), ammonium acetate, and N-(4-aminophenyl)piperidine were purchased from Sigma Aldrich (St. Louis, MO). Methanol (LC-MS grade), dimethylformamide (DMF), dichloromethane (DCM), N,N-diisopropylethylamine (DIPEA), sodium sulfate, formic acid, and acetic acid were purchased from VWR (Radnor, PA). Reagent grade ethyl acetate (EtOAc), hexanes, sodium bicarbonate, and sodium chloride were purchased from Greenfield Global (Brookfield, CT). HiPerSolv Chromanorm ethanol (90%, denatured with 5% isopropanol and 5% methanol) was also acquired from VWR. High-purity (instrument grade 4.0) carbon dioxide was purchased from Praxair (Danbury, CT). Buffered mobile phase modifiers were 10 mM ammonium acetate in methanol (or ethanol when noted, both adjusted to pH 5.5 with acetic acid) and 0.1% formic acid (v/v) in methanol.

2.2 Derivatization and Preparation of Organic Acid Samples

The general synthetic route is shown in **Figure 1**. Briefly, in a 20 mL screw cap vial equipped with a magnetic stir bar, each organic acid (0.142 mmol, mass variable depending on structure, 1.0 molar eq.) was dissolved in DMF (1.4 mL, 0.1M) and treated with DIPEA (3.0 molar eq. per carboxylic acid) and N-(4-aminophenyl)piperidine (1.0 molar eq. per carboxylic acid as a 0.5 M stock solution in DMF). This mixture was then treated with HATU (1.2 molar eq. per carboxylic acid) in a single portion. The resulting mixture was allowed to react at ambient temperature for 2 h and was transferred to a separatory funnel containing 30 mL DCM and 30 mL saturated aqueous $NaHCO_3$. The layers were separated and the aqueous layer was extracted with DCM (2 x 20mL). The combined organic layers were washed with saturated aqueous NaCl solution, were dried over Na_2SO_4 and concentrated *in vacuo*. The resulting crude mixture was purified to generate purer samples for further investigation by flash chromatography (Biotage Isolera, 60 μm silica, 25 g column) using a gradient of 20% EtOAc/hexanes to 100% EtOAc. Further details on the specific synthesis for each compound and further characterization details can be found in the *Supplementary Information*. Following derivatization and purification, individual compounds were dried by vacuum and reconstituted in methanol (except for citrate, which was reconstituted in DMSO to improve solubility) to a stock concentration of 5 mg/mL. Native, untagged organic acids were also prepared in stock solutions of 5 mg/mL. Further dilutions to reported concentrations were all made with methanol. All samples were filtered with a 0.2 μm syringe filter prior to injection.

2.3 Instrumentation, Columns, and Analysis

All experiments were performed using a Nexera UC SFC-MS system (Shimadzu, Kyoto, Japan) consisting of CO_2 and modifier solvent delivery pumps, autosampler, column oven, photodiode array (PDA) detector, backpressure regulator (BPR), make-up flow pump, and LCMS-2020 single quadrupole mass spectrometer. The column oven and BPR were set to 40ºC and 130 bar, respectively. Three columns were used in this study, all in a 3.0 x 150 mm format: HALO Penta-HILIC 5 μm (Advanced Materials Technology, Wilmington, DE), Chirex 3014 5 μm (Phenomenex, Torrance, CA), and Luna Omega Sugar 3 μm (Phenomenex, Torrance, CA). For analyte signal characterization, the Penta-HILIC column was equilibrated for 15 mins at the operating flow rate of 1.5 mL/min with a mobile phase consisting of 85% CO_2 and 15% methanol (with 10 mM ammonium acetate, pH 5.5). Injection volume was set at 2 μL and injections were performed in triplicate. Parameters for the LCMS-2020 used for MS detection for both native and derivatized organic acids are available in **Table S1**. Limit of detection (LOD), limit of quantification (LOQ) and sensitivity for each compound were evaluated via serial dilution of the native and derivatized organic acids over a range of 10 ng/mL (ppb) – 300 μg/mL (ppm). The LOD and LOQ were determined using the signal-to-noise ratio (S/N) at the lowest concentration measured to calculate the concentrations giving S/N ratios of 3 and 10, respectively, per previous published methodology used for similar analytes [14]. Signal was calculated as the max peak intensity above the baseline, and noise was the standard deviation of the baseline across 1 min near the peak. Data was processed in the instrument LabSolutions software (Shimadzu, Kyoto, Japan) and Microsoft Excel (Redmond, WA). Figures were generated using Igor Pro 6.0 (Wavemetrics, Lake Oswego, OR).

3. Results and Discussion

3.1 Observation of Organic Acid Signal Enhancement with Derivatization

Organic acids are a difficult class of metabolites to characterize by LC-MS or SFC-MS due to their high polarity and chemical functionality that typically requires negative ionization mode for detection. In this study, we focused on the detection of four common organic acids (lactic, succinic, malic, and citric) and compared signal intensity between native compounds and those that had been derivatized with a N-(4-aminophenyl)piperidine group. As shown in **Figure 2**, the peak signal for the two native organic acids that were detectable using SFC-MS, lactic acid and succinic acid, was less than 30,000 counts. Following derivatization, the peak signal is enhanced over 100-fold as the tag increases proton affinity and allows for higher intensity positive ionization. Peak signal calibration curves for native and derivatized compounds (**Figure 2**) demonstrate an average 430-fold increase in sensitivity for the compounds that were detectable in their native form. Calculated LODs, LOQs, and sensitivities for both compound sets based on these curves are shown in **Table 1**. The improvement in LOD varied from 22-fold for succinic acid to 3800-fold for lactic acid, with detection becoming possible for malic acid and citric acid post-derivatization. This is similar to previous observations in LC-MS for the derivatized compounds and demonstrates that the benefits of the N-(4-aminophenyl)piperidine tag extend to SFC-MS [13].

These plots were generated in selected ion monitoring (SIM) mode targeting the maximum peak identified in full scan mode (full scans shown in **Figure S1** and primary mass-to-charge ratios listed in **Table S2**). Even if multiple ionization sites were available in the native organic acids, the highest intensity signal was observed for the molecular ion $[M\text{-}H]^-$ in negative mode. For the derivatized compounds, the base peak varied with the number of tags, showing highest intensity for the molecular ion $[M+H]^+$ for singly tagged, $[M+K]^+$ for doubly tagged, and $[M+2H]^{2+}$ for triply tagged. Potassium ions can form metal ion clusters with alcohol modifiers to produce potassium adducts ($[M+K]^+$), which may result in ion suppression in SFC-MS [1,15]. Despite the spread of signal across multiple charge states and the presence of potassium adducts, the overall larger increase in signal intensity still permits a significant increase in sensitivity and improvements to detection limits for the derivatized compounds that could be useful in future metabolomic experiments using SFC-MS. In addition, multiple groups have reported difficulties measuring native organic acids using SFC-MS [8,9], which was also experienced here with malic and citric acids. With N-(4-aminophenyl)piperidine derivatization, these compounds can be identified and measured.

3.2 Effects of Modifier Additives and Make-Up Flow on Signal Intensity

Two key aspects of SFC-MS method development are the use of additives in the mobile phase modifier and the extent of make-up flow needed to ensure that the eluent stream is effectively delivered to the MS inlet following the backpressure regulator. For the analysis of derivatized organic acids, buffer additives can also play a crucial role in the ionization state of the compounds, affecting both their separation and detection [16,17]. In this study, the use of two common additives in SFC-MS, ammonium acetate (10 mM, pH 5.5) and formic acid (0.1% v/v), were investigated for their effect on a dicarboxylic acid (succinic acid) along with pure methanol as the primary organic modifier. For derivatized succinic acid, the use of ammonium acetate provided the highest signal intensity (**Figure 4**). With 0.1% formic acid, the doubly charged ion was more intense than the singly charged ion, but the signal of both charge states was suppressed relative to the other modifier conditions used. The effects of ammonium acetate were similar to previously observed effects on peak shape and signal intensity with this additive [18]. Because of the higher signal, preferential ionization to a single charge state, and improved peak shape, ammonium acetate was selected as the preferred additive to the methanol organic modifier for the derivatized compounds. One exception to this trend was citric acid, which had slightly higher intensity for the doubly charged peak ($[M+2H]^{2+}$) than the singly charged peak ($[M+H]^{+}$) (**Figure S1**). In general, the overall signal enhancement across the full range of compounds suggests ammonium acetate as the preferred additive.

Make-up flow in SFC-MS is typically used to promote ionization of analytes and better transport of the eluent stream following the backpressure regulator, where expansion of the carbon dioxide can begin to occur [19,20]. Because of the high proton affinity N-(4-aminophenyl)piperidine tag used for derivatization generally helping improve ionization, the effect of an additional 0.15 mL/min make-up flow (10% of 1.5 mL/min total mobile phase flow rate) on derivatized succinic acid was tested. As shown in **Figure 5**, the use of make-up flow did not increase the overall signal intensity, and actually decreased the signal when ammonium acetate was present in the make-up flow solvent. However, when the make-up flow included the ammonium acetate additive, the relative intensity of the singly charged ionization state to the doubly charged state was higher (**Figure S2**). Based on previous literature study, the loss of compressibility of CO_2 post-BPR may enhance precipitation of analytes and loss of chromatographic integrity, hence posing a significant challenge towards detection of the analytes by SFC-MS [20]. For these derivatized compounds, the ability to effectively detect them by SFC-MS without make-up flow enables simpler operation of the technique by requiring one less mobile phase pump.

3.3 Preliminary Column Screen for Separation of Derivatized Organic Acids

The primary focus of this study was the effect of N-(4-aminophenyl)piperidine derivatization on the MS signal intensity of organic acids under SFC elution conditions. However, for eventual implementation in SFC-MS metabolomics, conditions for analyte separation by SFC will also be needed. To that end, three columns were screened for analyte separation using SFC-MS. Maintaining the 15% methanol modifier with 10 mM ammonium acetate additive, the separation of the four derivatized organic acids on these columns is shown in **Figure 6**. All three columns demonstrated the same order of elution based on the number of derivatization moieties. The highest resolution for the derivatized dicarboxylic acids (typical critical pair) was obtained with the Penta-HILIC column while the overall highest retention for compounds was observed using the Chirex 3014 column (which had previously been used for compounds with similar structural characteristics to the derivatized organic acids [21]). Separation was further investigated using the Penta-HILIC column, with retention expectedly decreasing with increasing modifier amount from 10% to 20% (**Figure S3**). As ethanol is a common alternative to methanol as a modifier for SFC separations [22], its use was also tested (**Figure S4**). Ethanol is a weaker modifier than methanol when using polar stationary phases in SFC, so the retention increased significantly with ethanol, especially for tagged citric acid. The ethanol modifier also resulted in lower SFC-MS signal intensity, likely due to an increase in gas-phase proton affinity compared to methanol [23], leading to methanol as the preferred organic solvent for the separation and detection of the derivatized organic acids.

4. Conclusions

The separation and detection of organic acids can be a challenging analytical task, with various issues occurring with HILIC, SFC, and MS detection. Here, a strategy employing N-(4-

aminophenyl)piperidine of carboxylic acid groups in these molecules was demonstrated. The detection sensitivity of four commonly monitored compounds in this analyte class was increased significantly over native forms, providing an improved technique for their measurement. Further, analytes that weren't observed in native form (malic and citric acids) were easily measured by SFC-MS once derivatized. Further exploration of derivatized succinic acid identified ammonium acetate as an effective modifier additive and the capability to maintain analyte signal without SFC make-up flow, which is commonly used for SFC-MS methods. In the future, this derivatization strategy will be used for the analysis of these compounds using nanospray SFC techniques [24,25] to further enhance MS signal intensity.

Acknowledgements

The authors would like to thank Jason Anspach (Phenomenex) and Stephanie Schuster (Advanced Materials Technology) for generously providing the columns used in this study. This work was supported by funding from the Chemical Measurement and Imaging Program in the National Science Foundation Division of Chemistry to J.P.G. (CHE-1904454) and to J.L.E. (CHE-1904919).

References

[1] van de Velde, B., Guillarme, D., Kohler, I., Supercritical fluid chromatography – Mass spectrometry in metabolomics: Past, present, and future perspectives. *J. Chromatogr. B* 2020, 1161, 122444.

[2] Gowda, G. A. N., Zhang, S., Gu, H., Asiago, V., Shanaiah, N., Raftery, D., Metabolomics-based methods for early disease diagnostics. *Expert Rev. Mol. Diagn.* 2008, 8, 617–633.

[3] Tang, D. Q., Zou, L., Yin, X. X., Ong, C. N., HILIC-MS for metabolomics: An attractive and complementary approach to RPLC-MS. *Mass Spectrom. Rev.* 2016, 35, 574–600.

[4] McCalley, D. V., A study of column equilibration time in hydrophilic interaction chromatography. *J. Chromatogr. A* 2018, 1554, 61–70.

[5] Ngere, J. B., Ebrahimi, K. H., Williams, R., Pires, E., Walsby-Tickle, J., McCullagh, J. S. O., Ion-Exchange Chromatography Coupled to Mass Spectrometry in Life Science, Environmental, and Medical Research. *Anal. Chem.* 2023, 95, 152–166.

[6] Cech, N. B., Enke, C. G., Practical implications of some recent studies in electrospray ionization fundamentals. *Mass Spectrom. Rev.* 2001, 20, 362–387.

[7] Rao, W., Pan, N., Tian, X., Yang, Z., High-Resolution Ambient MS Imaging of Negative Ions in Positive Ion Mode: Using Dicationic Reagents with the Single-Probe. *J. Am. Soc. Mass Spectrom.* 2016, 27, 124–134.

[8] Desfontaine, V., Losacco, G. L., Gagnebin, Y., Pezzatti, J., Farrell, W. P., González-Ruiz, V., Rudaz, S., Veuthey, J. L., Guillarme, D., Applicability of supercritical fluid chromatography – mass spectrometry to metabolomics. I – Optimization of separation conditions for the simultaneous analysis of hydrophilic and lipophilic substances. *J. Chromatogr. A* 2018, 1562, 96–107.

[9] Sen, A., Knappy, C., Lewis, M. R., Plumb, R. S., Wilson, I. D., Nicholson, J. K., Smith, N. W., Analysis of polar urinary metabolites for metabolic phenotyping using supercritical fluid chromatography and mass spectrometry. *J. Chromatogr. A* 2016, 1449, 141–155.

[10] Herniman, J. M., Worsley, P. R., Greenhill, R., Bader, D. L., John Langley, G., Development of ultra-high-performance supercritical fluid chromatography-mass spectrometry assays to analyze potential biomarkers in sweat. *J. Sep. Sci.* 2022, 45, 542–550.

[11] Cech, N. B., Enke, C. G., Electrospray and MALDI Mass Spectrometry: Fundamentals, Instrumentation, Practicalities, and Biological Applications: Second Edition. 2012, pp. 49–73.

[12] Huang, T., Armbruster, M. R., Coulton, J. B., Edwards, J. L., Chemical Tagging in Mass Spectrometry for Systems Biology. *Anal. Chem.* 2019, 91, 109–125.

[13] Guan, S., Armbruster, M. R., Huang, T., Edwards, J. L., Bythell, B. J., Isomeric Differentiation and Acidic Metabolite Identification by Piperidine-Based Tagging, LC-MS/MS, and Understanding of the Dissociation Chemistries. *Anal. Chem.* 2020, 92, 9305–9311.

[14] Kloos, D., Derks, R. J. E., Wijtmans, M., Lingeman, H., Mayboroda, O. A., Deelder, A. M., Niessen, W. M. A., Giera, M., Derivatization of the tricarboxylic acid cycle intermediates and analysis by online solid-phase extraction-liquid chromatography-mass spectrometry with positive-ion electrospray ionization. *J. Chromatogr. A* 2012, 1232, 19–26.

[15] Haglind, A., Hedeland, M., Arvidsson, T., Pettersson, C. E., Major signal suppression from metal ion clusters in SFC/ESI-MS - Cause and effects. *J. Chromatogr. B* 2018, 1084,

96–105.

[16] Ovchinnikov, D. V., Ul'yanovskii, N. V., Kosyakov, D. S., Pokrovskiy, O. I., Some aspects of additives effects on retention in supercritical fluid chromatography studied by linear free energy relationships method. *J. Chromatogr. A* 2022, 1665, 462820.

[17] Liigand, J., Laaniste, A., Kruve, A., pH Effects on Electrospray Ionization Efficiency. *J. Am. Soc. Mass Spectrom.* 2017, 28, 461–469.

[18] Cazenave-Gassiot, A., Boughtflower, R., Caldwell, J., Hitzel, L., Holyoak, C., Lane, S., Oakley, P., Pullen, F., Richardson, S., Langley, G. J., Effect of increasing concentration of ammonium acetate as an additive in supercritical fluid chromatography using CO2-methanol mobile phase. *J. Chromatogr. A* 2009, 1216, 6441–6450.

[19] Liigand, J., de Vries, R., Cuyckens, F., Optimization of flow splitting and make-up flow conditions in liquid chromatography/electrospray ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* 2019, 33, 314–322.

[20] Akbal, L., Hopfgartner, G., Hyphenation of packed column supercritical fluid chromatography with mass spectrometry: where are we and what are the remaining challenges? *Anal. Bioanal. Chem.* 2020, 412, 6667–6677.

[21] Goetz, G. H., Farrell, W., Shalaeva, M., Sciabola, S., Anderson, D., Yan, J., Philippe, L., Shapiro, M. J., High throughput method for the indirect detection of intramolecular hydrogen bonding. *J. Med. Chem.* 2014, 57, 2920–2929.

[22] West, C., Lesellier, E., Effects of mobile phase composition on retention and selectivity in achiral supercritical fluid chromatography. *J. Chromatogr. A* 2013, 1302, 152–162.

[23] Gazárková, T., Plachká, K., Svec, F., Nováková, L., Current state of supercritical fluid chromatography-mass spectrometry. *TrAC - Trends Anal. Chem.* 2022, 149, 116544.

[24] Mostafa, M. E., Grinias, J. P., Edwards, J. L., Supercritical Fluid Nanospray Mass Spectrometry. *J. Am. Soc. Mass Spectrom.* 2022, 33, 1825–1832.

[25] Mostafa, M. E., Hayes, M. M., Grinias, J. P., Bythell, B. J., Edwards, J. L., Supercritical Fluid Nanospray Mass Spectrometry: II. Effects on Ionization. *J. Am. Soc. Mass Spectrom.* 2023, In Press.

Tables

Table 1. Figures of merit for native and derivatized organic acids (calculated using S/N ratio and peak height/intensity as described in the text). "N.D." denotes "not detected".

		LOD (ppb)	**LOQ (ppb)**	**Sensitivity (Counts/ppb)**
Native Compounds	**Lactate**	1900	6400	2
	Succinate	33	110	15
	Malate	N.D.	N.D.	N.D.
	Citrate	N.D.	N.D.	N.D.
Derivatized Compounds	**Lactate**	0.5	1.5	1290
	Succinate	1.5	5	3400
	Malate	2.5	8.5	450
	Citrate	9	30	37

Figure Captions

Figure 1. Reaction scheme for derivatization of carboxylic acid groups with N-(4-aminophenyl)piperidine.

Figure 2. Extracted ion chromatograms of derivatized (red trace) and native (blue trace) lactic acid (A, both 3 ppm) and succinic acid (B, both 1 ppm).

Figure 3. Calibration curves for the four derivatized (solid lines) and native (dashed lines) organic acids (dashed lines). Red circles are measured signals for lactate (A), blue circles are measured signals for succinate (B), black diamonds are measured signals for malate (C), and green triangles are measured signals for citrate (C). Error bars represent ±1 s.d. from triplicate injections. Native forms of malate and citrate were not characterized, so only data for the derivatized compounds is shown in panel C.

Figure 4. Mass chromatograms (SIM mode) and mass spectra for derivatized succinate using 15% methanol with 10 mM ammonium acetate pH 5.5 (black trace for molecular ion SIM, relative intensities of three primary ion SIMs in B) , no additive (red trace for molecular ion SIM, relative intensities of three primary ion SIMs in C), and 0.1% formic acid (blue trace for molecular ion SIM, relative intensities of three primary ion SIMs in D).

Figure 5. Mass chromatograms (SIM mode) at 451 m/z for derivatized succinate eluted using 15% methanol (w/ 10 mM ammonium acetate) modifier with no make-up flow (red trace), methanol

make-up flow (black trace), and methanol (w/ 10 mM ammonium acetate) make-up flow(blue trace).

Figure 6. Separation comparison of derivatized organic acids using 15% methanol modifier (w/ 10mM ammonium acetate, pH 5.5) for (A) HALO Penta-HILIC, (B) Phenomenex Luna Omega SUGAR, and (C) Phenomenex Chirex 3014.Compound concentrations for lactate (red trace), succinate (blue trace), malate (black trace), and citrate (green trace) were 200 ppb, 200 ppb, 1 ppm, and 10 ppm, respectively.

Figures

Figure 1.

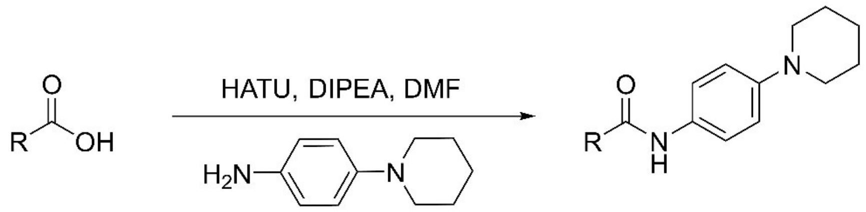

Figure 2.

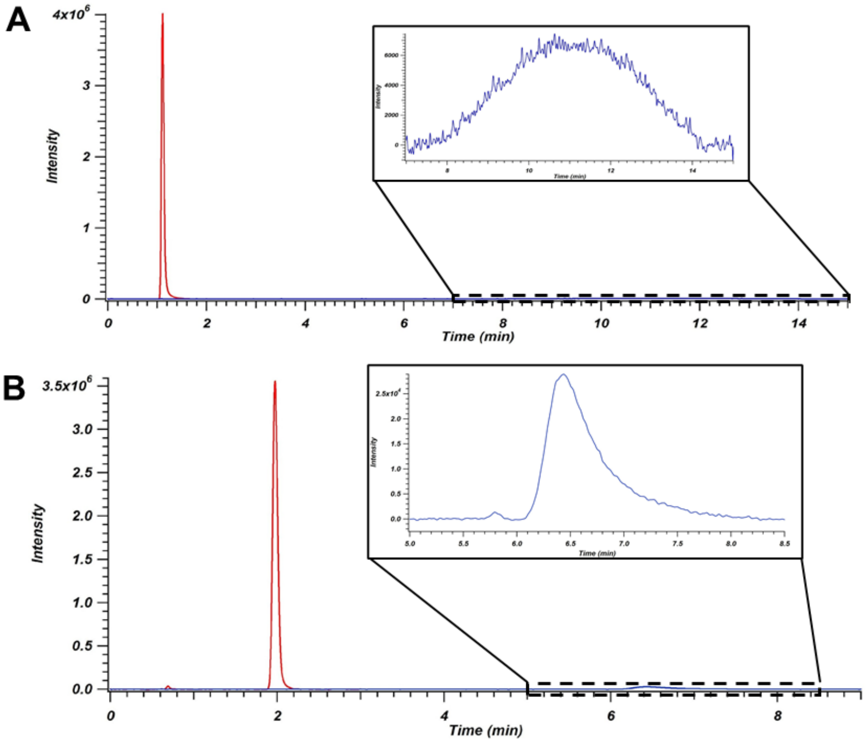

Figure 3.

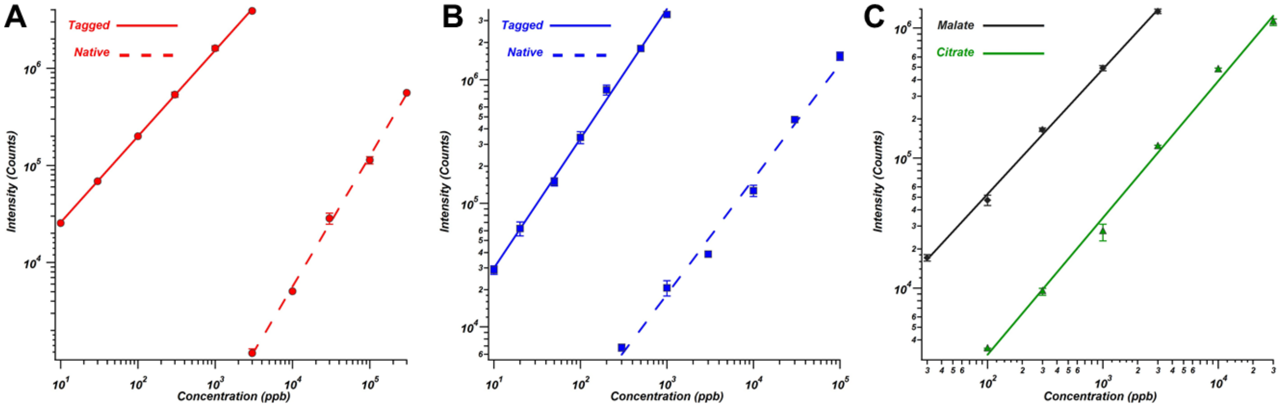

Figure 4.

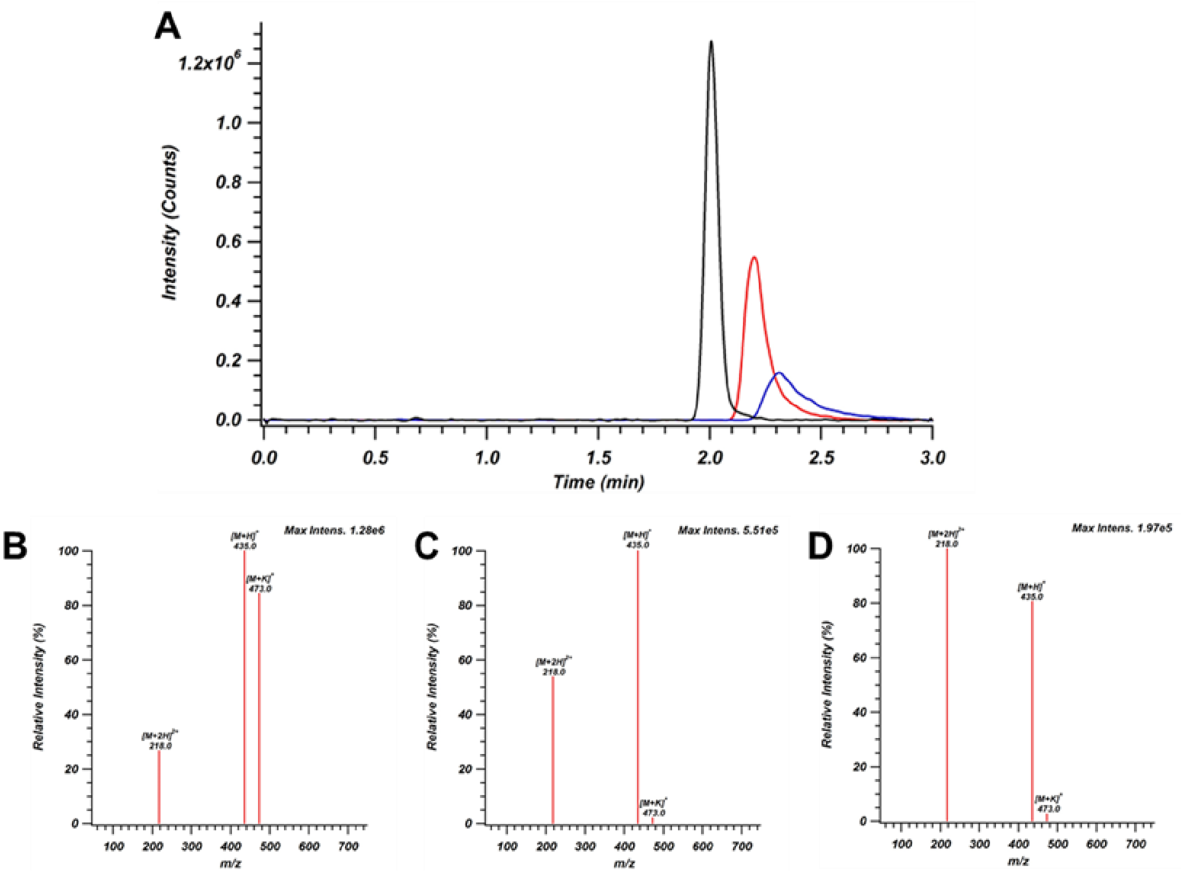

Figure 5.

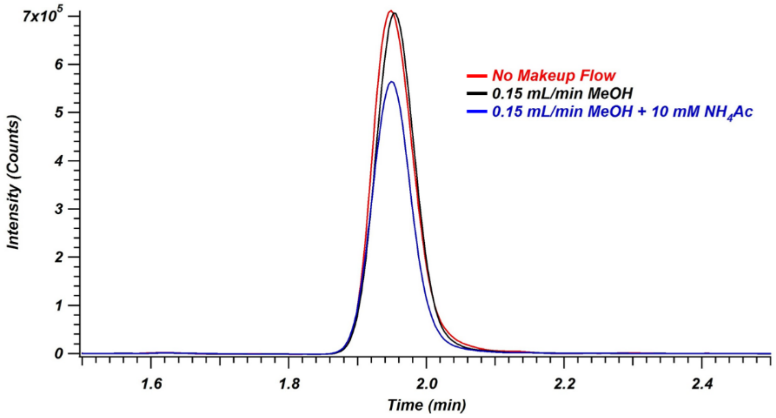

Figure 6.