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# Enhancement of Amino Acid Detection and Quantification by Electrospray Ionization Mass Spectrometry

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**A new strategy for amino acid analysis is reported involving derivatization with an *N*-hydroxysuccinimide ester of *N*-alkylnicotinic acid ($C_n$-NA-NHS) followed by reversed-phase chromatography and electrospray ionization mass spectrometry (RPC−MS). Detection sensitivity increased as the *N*-alkyl chain length of the nicotinic acid derivatizing agent was increased from 1 to 4. N-Acylation of amino acids with the $C_n$-NA-NHS reagents in water produced a stable product in roughly 1 min using a 4-fold molar excess of derivatizing agent in 0.1 M sodium borate buffer at pH values ranging from 8.5 to 10. Some O-acylation of tyrosine was also observed, but the product hydrolyzed within a few minutes at pH 10. The cystine product also degraded slowly over the course of a few days from reduction of the disulfide bond to form cysteine. The retention time of $C_n$-NA derivatized amino acids was lengthened in reversed-phase chromatography to the extent that polar amino acids were retained beyond the solvent peak, particularly in the cases of the $C_3$-NA and $C_4$-NA derivatives. Complete resolution of 18 amino acids was achieved in 28 min using the $C_4$-NA-NHS reagent. Compared to N-acylation with benzoic acid, derivatization with $C_4$-NA-NHS increased MS detection sensitivity 6−80-fold. This was attributed to the surfactant properties of the $C_n$-NA-NHS reagents. The quaternary amine increased the charge on amino acid conjugates while the presence of an adjacent alkyl chain further increased ionization efficiency by apparently enhancing amino acid migration to the surface of electrospray droplets. Further modification of the $C_n$-NA-NHS reagents with deuterium was used to prepare coded sets of derivatizing agents. These coding agents were used to differentially code samples and after mixing carry out comparative concentration measurements between samples using extracted ion chromatograms to estimate relative peak areas of derivatized amino acids.**

The evolution of methods for amino acid analysis has closely paralleled the development of analytical tools for the life sciences during the past 50 years. First there were the old paper chromatography methods, then the Moore and Stein ion exchange method with postcolumn ninhydrin-based detection[1] that led to HPLC, and finally reversed-phase chromatographic (RPC) separation methods with precolumn derivatization. Derivatization of amino acids with reagents such as *o*-phthaladehyde−mercaptoethanol,[2] dansyl chloride,[3] phenyl isothiocyanate,[4] and its analogues[5,6] fluorenylmethyl chlorofomate,[7] and 6-aminoquiline carbamate[8] vastly increased detection sensitivity.

Now mass spectral methods are playing a role in detection. Among the many advantages of mass spectrometry (MS) are that analytes do not have to be chromatographically resolved to allow detection. Another is that multiple dimensions of structure analysis are available through MS/MS analysis without adding time to the analysis. Moreover, extracted ion chromatograms allow multiple mass-related features of a mixture to be recognized, quantified, and displayed. This enables the detection of both parent ions and fragment ions common to multiple species.

But there are problems with the reversed-phase chromatography−mass spectrometry (RPC−MS) approach as well. One is that matrix effects can suppress the ionization of amino acids. This is particularly true of amino acids that do not ionize well in the first place. Yet another is that polar amino acids elute from RPC columns unretained in the column void volume with a large number of other substances. This problem has been addressed by derivatization with a more hydrophobic group such as benzoic acid[9] or 2,4-dinitrofluorobennzene (DNFB)[10] to increase RPC retention of amino acids. Unfortunately, derivatization can also impact ionization efficiency. Although all 19 DNFB derivatized amino acids examined were well resolved by RPC, only H, R, and K were observed in the positive ion mode of electrospray ionization (ESI)-MS.[11] This is apparently due to a loss in charge caused by derivatization.

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It is becoming clear that a strategy for dealing with poor ionization of amino acids is to derivatize them with a group that is easily protonated, such as a tertiary amine.[12] Dansyl derivatizes for example are more easily protonated during ESI because of the dimethylamino moiety in the naphthyl ring.[13] Derivatizing amino acids with dimethylformamide (DMF)−dimethylacetal also enhances detection in tandem mass spectrometry, especially for smaller amino acids such as glycine.[14] Derivatization with *N*,*N*-dimethyl-2,4-dinitro-5-fluorobenzylamine is another example in which a robust mass signal is obtained by introduction of a tertiary amine.[11] Quaternization could increase ionization efficiency even more as has been shown with steroids and peptides.[15−17]

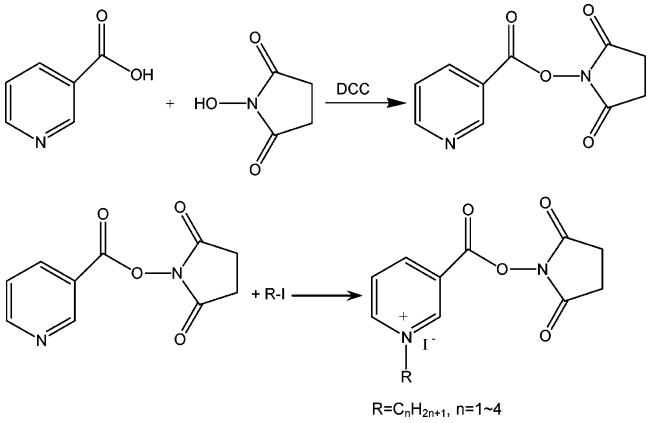
The objective of the work reported here was to explore the use of derivatizing agents to enhance RPC−MS analysis of amino acids by adding desirable features to analytes. One was to add sufficient hydrophobicity to cause the retention of amino acids well beyond the RPC solvent peak. A second was to add a quaternary amine group that would increase ionization efficiency. A third was to introduce stable isotope codes into amino acids to enhance relative quantification in comparative metabolomics. Derivatizing agents evaluated in these studies were obtained by synthesizing a family of *N*-alkylnicotinic acid derivatizing agents activated with *N*-hydroxysuccinimide.

## EXPERIMENTAL SECTION

**Materials and Reagents.** An amino acid standard mixture was obtained from Sigma-Aldrich (St. Louis, MO). The following amino acids including ammonium chloride were in the mixture: L-alanine, L-arginine, L-aspartic acid, L-cystine, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tyrosine, and L-valine. Amino acids in this standard mixture were at a concentration of 2.5 $\mu$mol/mL in 0.1 N HCl. The exception was L-cystine at 1.25 $\mu$mol/mL. Anhydrous acetonitrile (ACN), dicyclohexylcarbodiimide (DCC), alkyl iodide, *N*-hydroxysuccinamide, and DMF were also purchased from Sigma-Aldrich. HPLC grade acetonitrile and acetone were obtained from Mallinckrodt Baker (Phillipsburg, NJ). Trifluoroacetic acid (TFA) (Sequanal grade) was obtained from Pierce (Rockford, IL). 1-Iodobutane-$d_9$ was the product of Cambridge Isotope Laboratories (Andover, MA). Double-deionized water was produced by a Milli-Q gradient A10 system (Millipore, Bedford, MA).

**Synthesis of *N*-Hydroxysuccinimide-Activated *N*-Alkylnicotinic Acid Esters ($C_n$-NA-NHS).** The scheme for synthesizing *N*-alkylnicotinic acid *N*-hydroxysuccinimide ester ($C_n$-NA-NHS) derivatizing agents is shown in Scheme 1. The procedure is an adaptation of a method first described by Srivastava.[18] An aliquote of diclyclohexyl carbodiimide (DCC) (44 mmol, 9.0 g) was added to 5 mL of dry DMF containing 40 mmol (5.0 g) of nicotinic acid and 50 mmol of *N*-hydroxysuccinimide (4.6 g). The reaction mixture was stirred at room temperature for 48 h and then treated with glacial acetic acid (0.2 mL) to decompose excess DCC. After allowing the mixture to stir at room temperature for an additional hour, the temperature was reduced to 3−4 °C and stirring continued for another 4 h. Precipitated dicyclohexylurea was removed by filtration and the filtrate evaporated under vacuum to reduce the volume to ~10 mL. A pale yellow product was precipitated by addition of a mixture of hexane and 2-propanol (6:1 in volume). The nicotinic acid *N*-hydroxysuccinimide ester precipitate was dried under vacuum and used to prepare a series of *N*-alkylnicotinic acid *N*-hydroxysuccinimide esters (alkyl = $C_1$, $C_2$, $C_3$, $C_4$) by direct quaternization. A 5-mmol aliquot of nicotinic acid *N*-hydroxysuccinimide ester was added to 10 mmol of an alkyl iodide in 5 mL of ACN. The mixture was refluxed at 70−75 °C under argon for 24−48 h. A yellow product precipitated from the solution either during the reaction or after removing some of the solvent, depending on the alkyl groups. After cooling, the $C_n$-NA-NHS product was collected by filtration, washed with cold acetone, and dried to yield yellow crystals that were stored in a desiccator until used for amino acid derivatization.

**Scheme 1. Synthesis of *N*-Alkylnicotinic Acid *N*-Hydroxysuccinimide Ester ($C_n$-NA-NHS)**




**Derivatization of Amino Acids.** A $C_n$-NA-NHS solution was prepared at a concentration of 40.0 mg/mL in dry ACN. This solution is stable for 5 days at ambient temperature or at least one month at 4 °C. Two types of standard solutions were used in optimization studies. One type contained 50.0 $\mu$mol/mL of an individual amino acid in 0.1 mol/L hydrochloride. Tryptophan was an exception in that it was dissolved in deionized water instead of HCl. The other type of standard solution was made by adding 10.0 $\mu$L of all the individual amino acid solutions in HCl and 0.5 $\mu$L of 50.0 $\mu$mol/mL tryptophan in deionized water to 200 $\mu$L of 0.1 mol/L sodium borate buffer at pH 9.0. After mixing, 20 $\mu$L of 40.0 mg/mL $C_n$-NA-NHS (roughly a 4-fold molar excess) was added. The reaction was allowed to proceed for 1 min before RPC analysis. Samples of unknown amino acid concentration were analyzed by adding 10.0 $\mu$L of sample and 20 $\mu$L of 40.0 mg/mL $C_n$-NA-NHS solution to 200 $\mu$L of 0.1 mol/L sodium borate buffer at pH 9.0. After a minimum of 1-min incubation, derivatized amino acids were analyzed by RPC−MS. Incubation times of 10 min were used when it was the objective to allow O-acylated tyrosine to hydrolyze to a single N-acylated product.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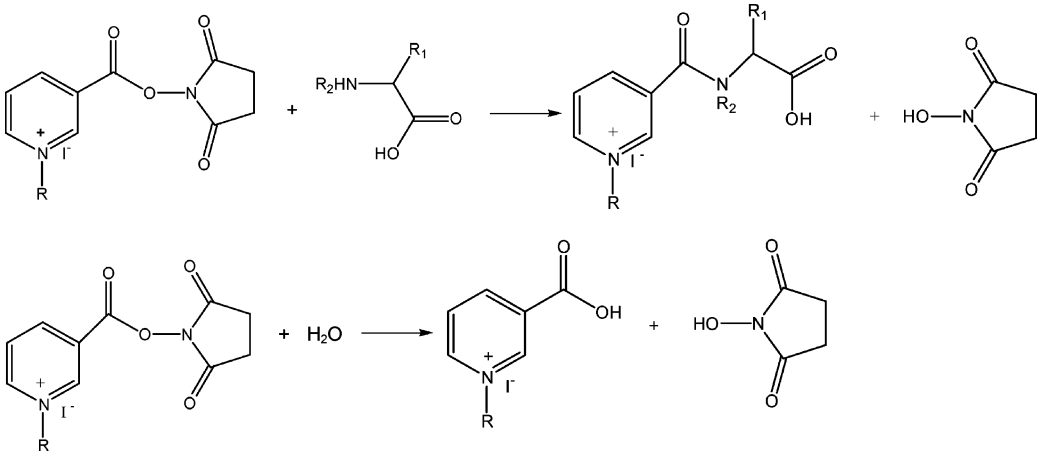
**Scheme 2. Reactions Involved in the Derivatization of Amino Acids with $C_n$-NA-NHS and Subsequent Hydrolysis of the Unused Derivatizing Agent[a]**



[a] Amino acid derivatization occurs rapidly. Hydrolysis of $C_n$-NA-NHS in contrast is a much slower reaction.

**RPC−ESI-MS Analysis.** Optimization of the derivatization reaction was achieved using RPC on an Integral Micro-Analytical workstation with a 205-nm-wavelength UV detector (Applied Biosystems, Framingham, MA). Amino acid derivatives were also purified with this system for direct MS analysis in some cases. RPC with ESI mass spectral analysis of amino acid derivatives was carried out on a Waters Alliance system, equipped with MassLynx 4.0 software, a Waters 996 photodiode array detector (PAD), and a Micromass Q-Tof micromass spectrometer. The PAD was used in the scanning mode to detect analytes between 200 and 400 nm. Positive ion mass spectra were acquired in the continuous mode. Ionization was achieved with an electrospray voltage of 3 kV, cone voltage of +30 V, and cone and desolvation gas flows of 5−8 and 500 L/h, respectively. The source block and desolvation temperatures were maintained at 150 and 350 °C, respectively. All separations were performed on a low TFA Vydac analytical column (C18 Mass Spec, 4.6 mm × 250 mm) using 15 $\mu$L of sample and gradient elution with two sets of mobile phases. Optimization of derivatization was achieved by eluting the RPC column initially with 5% ACN and 0.1% TFA in water for 5 min and then proceeding on in a 30-min linear gradient to 5% water and 0.1% TFA in ACN. General analyses were achieved with a second elution protocol using two different eluting solvents. Solvent A was composed of 0.05% TFA in water, and solvent B contained 5% $H_2O$ and 0.05% TFA in ACN. After 4 min of isocratic elution with solvent A, an 11-min linear gradient to 10% solvent B was initiated, followed by a 5-min linear gradient to 20% mobile phase B, and finally a 10-min linear gradient to 50% solvent B. The flow rate was 1 mL/min in all cases.

**ESI-MS/MS Analysis.** ESI-MS and ESI-MS/MS analysis was performed on an API QSTAR Pulsar LC/MS/MS System (Applied Biosystem, Framingham, MA) equipped with an ESI ion source. Fractions from the Intergral LC were collected, dried, reconstituted with $CH_3OH/H_2O$/acetic acid (50%/49%/1%), and injected into the ESI source by infusion at 5−10 $\mu$L/min. Typical settings for analysis in the positive mode of ESI were as follows: ion spray voltage, 5000; curtain gas, 20; ion source gas 1, 20; ion source gas 2, 0; declustering potential, 45; focusing potential, 220; declustering potential 2, 20.0. Collision energy was optimized to obtain maximum fragmentation for tandem MS.

## RESULTS AND DISCUSSION

**Amino Acid Derivatization.** Amino acid (AA) derivatization was achieved by amide bond formation through nucleophilic displacement of NHS from an *N*-alkylnicotinic acid (NA) by the α-amino group of the AA (reaction Scheme 2). In the case of lysine, the ϵ-amino group is sufficiently nucleophilic at slightly basic pH that it too is derivatized. Beyond being easily displaced, a major advantage of NHS activation is that derivatization can be achieved in water. This is a great benefit because amino acids are water soluble. A similar protocol for α-amino group derivatization is used with peptides where it has been shown that NHS activated nicotinic acid is sufficiently stable in water to quantitatively acylated primary amine groups before finally hydrolyzing.[19] The reagent used in these studies differs from the one used in peptide derivatization only in the addition of alkyl groups ($C_n$) to nicotinic acid (NA) and quaternization of the ring nitrogen. *N*-Alkylnicotinic acid reagents activated with NHS will be represented by the general formula $C_n$-NA-NHS throughout the rest of the paper. Hydrolysis of residual $C_n$-NA-NHS after derivatization can be accelerated by adjusting to pH 10 with hydroxlamine.[20] Hydrolysis is achieved in less than 1 min under these conditions to yield $C_n$-NA according to MS analysis. $C_n$-NA-NHS derivatizing agents were stored in acetonitrile to preclude premature hydrolysis.

Optimization of derivatization was examined using the $C_3$-NA-NHS reagent and tryptophan (Trp) with RPC−MS analysis of the reaction products. Peaks eluting at 7.5, 13.1, and 16.8 min were found to be of $m/z$ 166.1(+1), 204.2(+1), and 352.1(+1), respectively. The number in parentheses behind the $m/z$ value is the charge state of the ion observed in the MS spectrum. These three peaks were identified as $C_3$-NA, free Trp, and $C_3$-NA-Trp, according to their retention times and $m/z$ values. The derivatization reaction was investigated by carrying the reaction out in 200 $\mu$L of 0.1

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mol/L sodium borate buffer at pH 8.0 to which 10 μL of a 50 μmol/mL tryptophan solution was added along with 20 μL of derivatization reagent. $C_3$-NA-NHS and Trp were mixed in 1:1 and 3:1 molar ratios, and the reaction was allowed to proceed for 5 min. Although some Trp was seen at the 1:1 $C_3$-NA-NHS/Trp ratio, at the 3:1 molar ratio, Trp derivatization was complete in 30 s. A 1-min reaction time and 4:1 molar ratio were used in subsequent experiments to ensure that derivatization would be complete.

Further optimization of the reaction was carried out with a standard 17-amino acid mixture from Sigma (see Experimental Section) using the $C_3$-NA-NHS derivatizing agent. Yields were studied using 0.1 M sodium borate buffers ranging in pH from 7.5 to 10. Although the amount of product formed increased slightly between pH 7.5 and 8.5, there was little additional increase in product above pH 8.5. Similar findings have been reported with other acylating agents. [9,21] In addition to N-acylation, Tyr showed O-acylation in the pH range from 7.5 to 8.5. Three peaks containing Tyr were seen in the RPC tracing. One of the Tyr products was the expected α-amino group acylation product. Another was from acylation of the phenolic OH. O-Acylation of tyrosine has also been reported in the case of peptides where O-acylated products were hydrolyzed after derivatization by the addition of hydroxylamine.[20] The third arose from derivatizing both the phenolic hydroxyl and the α-amino group. At pH 7.5, the phenolic hydroxyl derivative was dominant with roughly 95% of the derivatization occurring at this site. At pH 8.0, greater than 90% of the Try product resulted from double derivatization. When the reaction was carried out at pH 9.0, the α-amino derivatized product dominated the mixture. These data were interpreted to mean that derivatization of the phenolic hydroxyl group occurred faster than at the α-amino group but was also being hydrolyzed. Given enough time at the higher pH, the exclusive product at pH 9.0 should be from derivatization on the α-amino group. This was in fact the case. After 10-min incubation at pH 9.0, the exclusive product was from α-amino group derivatization. Neither buffer concentration nor reagent-to-amino acid ratio had much influence on product distribution and yield under these conditions. As a consequence of the additional complication added by Tyr, derivatization reactions were carried out in 0.1 M sodium borate at pH 9.0 for 10 min at a reactant-to-analyte ratio of no less than 4:1. Although higher pH also favors hydrolysis of NHS esters, the rate of derivatization exceeded the rate of hydrolysis in the early phase of the reaction.

Reproducibility of derivatization was studied by analyzing the standard mixture 10 times according to the procedure described in the Experimental Section. Concentration was determined using peak areas derived from absorbance detection. The results of these analyses are presented in Table 1 as relative standard derivations. Reproducibility was generally better than 5%.

Derivative stability was examined by measuring UV peak area in reversed-phase chromatograms at 205 nm over one week. Amino acids acylated on primary amine groups with $C_3$-NA were found to be stable for one week at 4 °C or less. But there were additional complications with Met and Cyn. Peak areas for these amino acids could decrease up to 20% in one week of storage at ambient temperature. Cyn degradation was the result of disulfide bond cleavage with the formation of Cys (*m/z* 308.1(+1)). Cys elutes between Asp and Arg in RPC. Degradation products for Met were not identified. Tyr O-acylation is also a problem. Generally it would be recommended to remove O-acylation by hydrolysis before either analysis or storage before analysis. Hydrolysis of the O-acylated Tyr derivative accelerates with increasing pH. It is for this reason that this product is easily destroyed by addition of hydroxylamine to achieve a slightly basic pH.[21] Hydroxylamine treatment of samples before analysis is thus a good idea.

**Table 1. *m/z* Value, Retention Time, and Reproducibility of Derivatization (RSD of peak area) for $C_4$-NA Derivatives of Amino Acids**

| amino acid | *m/z* | retention time (min) | peak area RSD (%, $n = 10$)[a] |
|---|---|---|---|
| His | 317.02 (+1) | 11.89 | nd[b] |
| Ser | 267.01 (+1) | 13.25 | 1.3 |
| Gly | 237.02 (+1) | 13.83 | 1.8 |
| Asp | 295.00 (+1) | 14.18 | 4.2 |
| Arg | 336.05 (+1) | 14.64 | 2.3 |
| Glu | 309.01 (+1) | 16.34 | 3.3 |
| Pro | 277.04 (+1) | 16.67 | 4.5 |
| Thr | 281.03 (+1) | 17.16 | 3.8 |
| Ala | 251.04 (+1) | 17.55 | 1.6 |
| Tyr-OH | 343.01 (+1) | 19.21 | nd |
| Tyr-$NH_2$ | 343.03 (+1) | 23.03 | 3.5 |
| (di-) Lys | 469.13 (+1) | 23.31 | 2.4 |
| | 235.07 (+2) | | |
| Val | 279.05 (+1) | 23.64 | 2.6 |
| (di-) Cyn | 562.01 (+1) | 23.84 | 4.4 |
| | 281.51 (+2) | | |
| Met | 311.01 (+1) | 24.00 | 4.6 |
| Tyr-OH, $NH_2$ | 504.09 (+1) | 25.15 | nd |
| | 252.55 (+2) | | |
| Ile | 293.05 (+1) | 25.63 | 1.1 |
| Leu | 293.05 (+1) | 26.08 | 1.0 |
| Phe | 327.02 (+1) | 26.52 | 1.3 |
| Trp | 366.02 (+1) | 26.88 | 1.9 |

[a] From UV absorbance at 205 nm. [b] nd, not detected.

**Reversed-Phase Chromatography.** Hydrophilic amino acids do not interact with the hydrophobic stationary phase of an RPC column, typically eluting in the column void volume. One of the objectives in this work was to circumvent this problem by increasing the hydrophobicity and concomitantly the RPC retention time of amino acids through derivatization. It was for this reason that alkyl groups were added to nicotinic acid. The experiments below were based on the rationale that by varying the aliphatic chain length in $C_n$-NA derivatizing agents it should be possible to manipulate the retention times of amino acids widely in the reversed-phase chromatogram. It will be shown later in the paper that extending the aliphatic chain length of $C_n$-NA also increased detection sensitivity.

It is seen in Figure 1 that the RPC retention time of even the most hydrophilic amino acids could be increased to greater than 10 min by derivatization. With the $C_1$-NA-NHS reagent, only Ile, Leu, Phe, and Trp were detected. This reagent did not increase hydrophobility of the hydrophilic amino acids sufficiently to retain them beyond the trailing solvent peak. As the alkyl chain length was increased to 3, all 18 amino acids were adequately retained to be detected. $C_4$-NA-NHS was used in subsequent reactions because detection sensitivity was higher with $C_4$-NA derivatization.

The RPC separation of 18 $C_4$-NA derivatized amino acids within 27 min is seen in Figure 2. The derviatization time in this case

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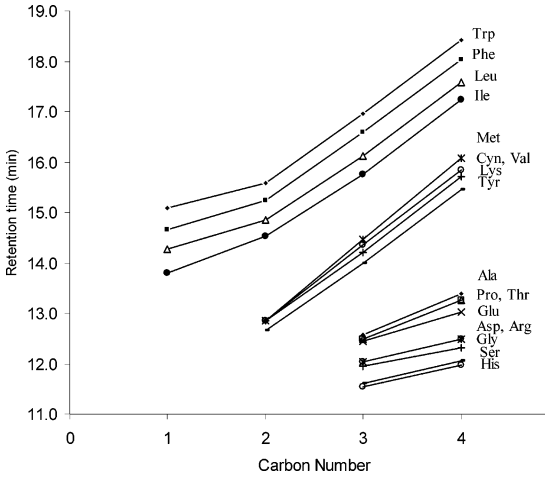


**Figure 1.** Effects of alkyl chain length on retention time of derivatized amino acids. Buffer A, 100% $H_2O$ + 0.1% TFA; buffer B, 95% ACN + 0.1% TFA. Elution protocol: 0−5 min, 100% A; 5−30 min, 100% A−100% B.

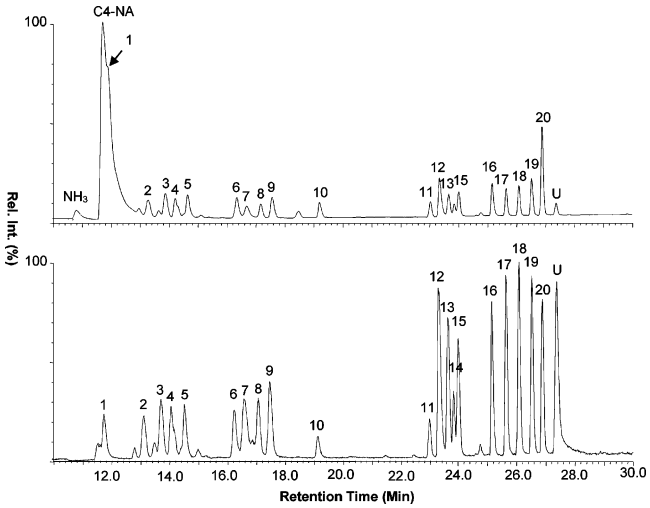


**Figure 2.** Reversed-phase chromatogram from 18 amino acids derivatized with $C_4$-NA-NHS using 1-min incubation time. The upper trace was obtained with photodiode array detection from 200 to 400 nm. The lower trace was obtained by extraction ion monitoring from *m*/*z* 190 to 600. Elution of the RPC columns was achieved with a two-solvent gradient. Buffer A was 0.05% TFA in water while buffer B contained 0.05% TFA in ACN. The gradient protocol was as follows: 0−4 min, 100% A; 4−15 min, 0% B−10% B; 15−20 min, 10% B−20% B; and 20−30 min, 20% B−50% B. Peak identification: 1, His; 2, Ser; 3, Gly; 4, Asp; 5, Arg; 6, Glu; 7, Pro; 8, Thr; 9, Ala; 10, Tyr-OH; 11, Tyr-$NH_2$; 12, Bis-Lys; 13, Val; 14, Cyn; 15, Met; 16, Bis-Tyr; 17, Ile; 18, Leu; 19, Phe; 20, Trp; U, unknown.

was 1 min. Peaks were assigned based on their unique *m*/*z* values except for $C_4$-NA-Ile and $C_4$-NA-Leu, which have identical masses and showed the same singly charged ion at *m*/*z* 293.05(+1). They were distinguishable by both their chromatographic retention times and tandem mass spectra. $C_4$-NA-His was obscured in the photodiode array trace by $C_4$-NA arising from the hydrolysis of $C_4$-NA-NHS. However, it was easily recognized in the extracted total ion chromatogram (TIC trace) when the *m*/*z* range was set for 190−600. But there is a penalty from excluding ions under *m*/*z* 190. Although $C_4$-NA appears at *m*/*z* 180.0 and is eliminated from the spectra, ammonia is also derivatized under these conditions and gives a distinct chromatographic peak. Unfortunately this product is excluded as well.

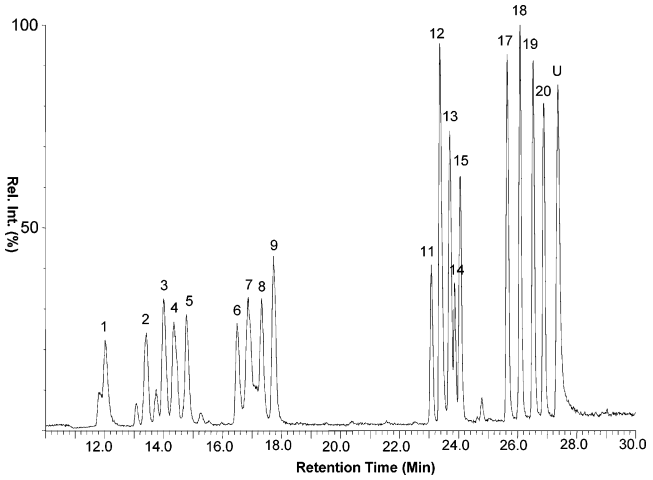


**Figure 3.** Extracted total ion chromatogram from 18 amino acids derivatized with $C_4$-NA-NHS using 10-min incubation time. Detection was achieved by monitoring the *m*/*z* 190−600 range. Separation conditions and peak identification are the same as in Figure 2.

In addition to N-acylation, O-acylation of tyrosine (peak 10) and bistyrosine (peak 16) was observed with a short derivatization time of 1 min (Figure 2). As the derivatization time was increased from 1 to 10 min, it is clearly seen in Figure 3 that these peaks are no longer present. Although phenolic hydroxyl groups derivatize rapidly, they are not stable in water and hydrolyze during a 10-min incubation.

One of the advantages of $C_n$-NA derivatization is that it also facilitates detection by UV absorbance through the addition of the aromatic ring in nicotinic acid. It is seen from Figure 2 that the UV response of nonaromatic amino acids was very similar with the exception of Lys and cystine (Cyn). These amino acids were doubly labeled by $C_n$-NA-NHS and thus have one more nicotinic acid residue than the other nonaromatic amino acids. Nicotinic acid residues show weak absorbance at 265 nm and absorb strongly below 210 nm.

**Enhancement of Ionization**. The requisite acylation of amino groups in amino acids to achieve derivatization reduces their charge. Concomitantly, this is expected to decrease ionization efficiency in positive ionization mode ESI-MS. The impact of charge on ionization efficiency of derivatized amino acids was examined using nicotinoate and benzoate derivatized Glu, Try, and Lys. $^{13}C_6$ Isotopically coded benzoyl derivatives (BA-$^{13}C_6$) of Glu, Trp, and Lys were prepared using methods described in the literature.[9] Use of BA-$^{13}C_6$ was necessary because benzoate and nicotinoate vary by only 1 atomic mass unit. Equal amounts of these three amino acids were derivatized individually with $^{13}C_6$-BA-NHS and NA-NHS, respectively, and examined individually by RPC. Peaks of analyte were collected, dried, and redissolved in 50% methanol + 49% $H_2O$ + 1% acetic acid (v/v/v). Equal aliquots of the six derivatives were mixed with the exception of BA-$^{13}C_6$-Glu, which was added at five times the volume of the others. An ESI-MS analysis of the mixture was performed as described in the Experimental Section. Relative differences in detection sensitivity are summarized in Table 2. Derivatization with nicotinic acid was seen to enhance detection sensitivity with Glu and Try, but not Lys. This is attributed to the ionization of nitrogen in the pyridine ring.

**Table 2. Comparison of Relative MS Signal Intensity for Amino Acids Derivatized with Different Reagents[a]**

| amino acid | derivatizing reagent | | | | | |
|---|---|---|---|---|---|---|
| | $C_1$-NA-NHS | $C_2$-NA-NHS | $C_3$-NA-NHS | $C_4$-NA-NHS | NA-NHS | BA-$^{13}C_6$-NHSS |
| Glu | nd[b] | 1.6 | 3.1 | 3.3 | 1.0 | 0.11 |
| Lys | 2.3 | 3.3 | 5.0 | 6.3 | 1.0 | 0.96 |
| Trp | 2.5 | 3.9 | 4.8 | 12.5 | 1.0 | 0.15 |

[a] Data were from two sets of experiments. See the text. Signal intensities were normalized to NA-NHS = 1.0. [b] nd, not detected.

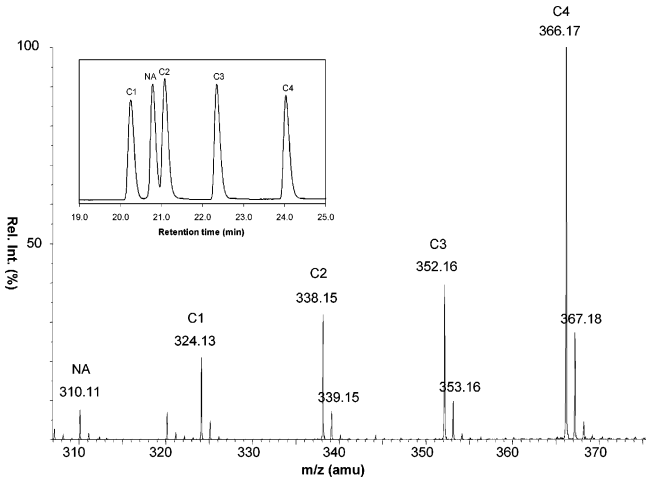


**Figure 4.** Mass spectra of NA- and $C_1$∼4-NA derivatives of tryptophan. The inset is the reversed-phase chromatogram of the NA- and $C_{1-4}$-NA derivatives of tryptophan with UV detection at 204 nm. All derivatives were of the same concentration.

A more dramatic enhancement of sensitivity was observed for the amino acids derivatized through permanently charged and alkylated derivatizing reagents $C_{1-4}$-NA-NHS. The $C_{1-4}$-NA-Trp derivative mixture along with NA-Trp was prepared and examined by ESI-MS. Although UV absorbance of the $C_{1-4}$-NA-Trp derivatives was very similar, response in ESI-MS was substantially larger with the longer alkyl chain length derivatives (Figure 4). Compared to nicotinoate derivatized tryptophan (NA-Trp), $C_4$-NA-Trp gave 12.5 times greater MS signal intensity. Comparisons were also made with Glu and Lys (Table 2). Although the degree of enhancement was not as great with these amino acids, even with Glu signal intensity was more than 3 times larger.

The results in Table 2 can be explained in the following way. Clearly increasing charge in the pyridine ring through quaternization enhances sensitivity as seen by the difference in response between NA-AA and $C_1$-NA-AA. This phenomenon has also been noted in the literature with peptides.[16,17] But this does not explain how longer alkyl chains further enhance sensitivity. It has been noted in ESI-MS that during the ionization process species that are both hydrophobic and cationic migrate to the droplet surface. When present at high concentration, they even suppress the ionization of less hydrophobic cationic species by forcing them away from the droplet surface. In view of the fact that ionization is thought to occur from droplet surfaces in ESI-MS, this process has been offered as an explanation of ion suppression. Based on this model of ESI-MS, it is likely that lengthening the alkyl chain in $C_{1-4}$-NA-AA causes the derivatized amino acid to have more

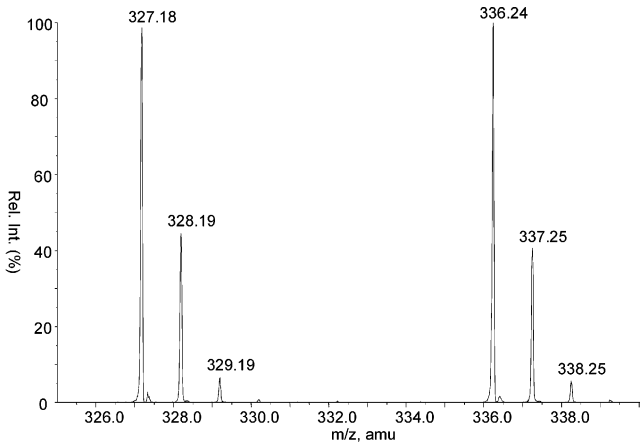


**Figure 5.** Mass spectra of $C_4H_9$-NA-Phe and $C_4D_9$-NA-Phe at a 1:1 molar ratio.

surfactantlike properties. This in turn increases AA concentration at droplet surfaces in ESI-MS and enhances ionization. The net effect is that, in comparison to BA-$^{13}C_6$ derivatives, $C_4$-NA derivatives of Glu, Lys, and Trp showed 30, 6, and 83 times greater ESI-MS signal intensity, respectively.

**Comparative Quantification through Deuterium Labeling.** In vitro stable isotope coding is now widely used in proteomics for quantitative comparisons of peptide and protein concentration between samples. The most successful of these approaches is to acylate α- and ϵ-amino groups of polypeptides in a sample with a reagent that allows global coding of all primary amine-containing species according to sample origin. When these differentially coded samples are then mixed and analyzed by ESI-MS, the spectra contain doublet clusters of ions from the coded peptide isotopomers. The difference in mass between the doublets is simply the stable isotope mass differential between the isotopically coded labeling agents. The difference in peak area of the clusters is proportional to the relative concentration of the peptide in the two samples. The relative concentration of thousands of peptides can be determined in a single RPC−MS in this in vitro coding method with a relative standard deviation of roughly 6%.[22]

A similar approach to comparative quantification was examined in this work. A heavy form of $C_4$-NA-NHS was synthesized by substituting $D_9$-*n*-butyl iodide for $H_9$-*n*-butyl iodide in the NA-NHS alkylation reaction, producing $C_4D_9$-NA-NHS that is 9 atomic mass units (amu) higher than the light form of the coding agent, $C_4H_9$-NA-NHS. Phenylalanine (Phe) was used for initial ESI-MS comparative quantification studies. $C_4D_9$-NA-Phe and $C_4H_9$-NA-Phe were mixed at concentration ratios varying over a 400-fold range in concentration. Isotope ratio analyses were performed by infusing samples into the ESI-MS through a nanospray inlet. The characteristic double cluster of ions separated by 9 amu is seen in the spectrum of a 1:1 concentration ratio sample (Figure 5). The isotope ratio calibration curve was found to be linear over a 400-fold range of concentration with a linearity coefficient ($r^2$) of 0.988 (data not shown). These data suggest that comparative quantification of amino acids by stable isotope coding will be possible using ESI-MS.

A more extensive study of comparative quantification was undertaken by differentially coding samples containing the entire

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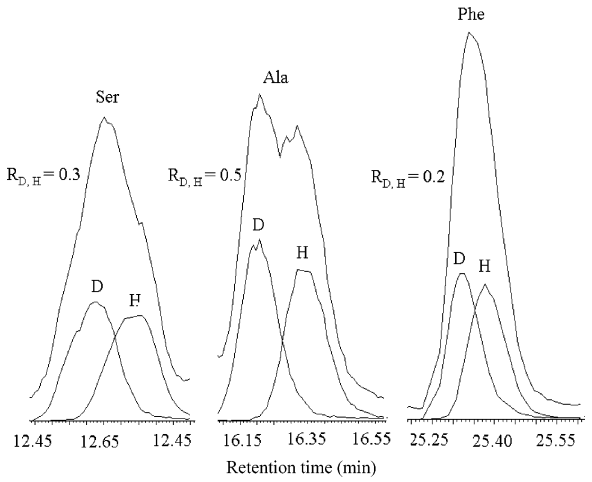


**Figure 6.** Chromatographic isotope effects for three amino acids derivatized with $C_4H_9$-NA-NSH and $C_4D_9$-NA-NSH at the ratio of 1:1. D = deuterium-labeled derivatives; H = nonlabeled derivatives. $R_{D,H}$ refers to resolution of the heavy and light isotope-labeled derivatives. Experimental conditions as in Figure 3.

set of amino acid standards. These sample mixtures were prepared by splitting a mixture of amino acid standards into equal aliquots; i.e., the amino acid concentration ratio between the two samples was 1:1. When amino acids in these two samples were differentially coded with the $C_4D_9$-NA-NHS and $C_4H_9$-NA-NHS labeling agents, respectively, and analyzed by RPC−MS, it was seen in multiple cases that the isotopomers of amino acids did not coelute (Figure 6). This meant that isotope ratio analysis could only be achieved by comparing the chromatographic peak area of amino acid isotopomers obtained from extracted ion chromatograms constructed after the analysis of the mixture was finished. Real-time isotope ratio analysis is precluded in cases were amino acid isotopomers are partially resolved because the isotope ratio at any single point in time does not reflect that in the initial mixture. This chromatographic isotope effect is common with deuterated isotopomers.[23] It has also been noted with peptide isotopomers that the chromatographic isotope effect can be circumvented by placing deuterium atoms in the coding agent beside a quaternary amine or by using $^{13}C$ coding instead of $^{2}H$.[24]

Clearly stable isotope-based quantification can be of value in metabolomics and other fields where it is the objective to compare the concentration of amino acids between samples. The issue is whether the chromatographic isotope effect has an impact on the determination of isotope ratios. Put another way, if there were no chromatographic isotope effect, which would provide the greatest accuracy: (1) comparison of peak areas from an extracted ion chromatogram or (2) a statistically based comparison of 10−50 direct MS-based isotope ratio measurements. The only way to test this is to prepare a $^{13}C$ coded labeling agent that shows no chromatographic isotope effects. Preparation of such a reagent is in progress.

## CONCLUSIONS

It is concluded that derivatization of amino acids by N-acylation with an acid that is also hydrophobic and has a quaternary amine can facilitate RPC/MS analysis of amino acids in several ways. One is by increasing the retention of small hydrophilic amino acids during reversed-phase chromatography sufficiently to cause them to elute beyond the void volume peak. This is necessary to resolve them from large amounts of other interfering polar, nonretained substances in complex samples. A second contribution is to aid in electrospray ionization by increasing the charge on amino acids through introduction of a quaternary amine group. A third way is by combining the effect of having both a hydrophobic and quaternary amine groups in proximity. Lengthening the alkyl chain in the hydrophobic quaternary amine portion of derivatized amino acids increases their surface-active properties and directs them to the surface of electrospray droplets where ionization is more likely to occur.

It is further concluded that during the course of in vitro derivatization of amino acids to increase their ionization efficiency the opportunity arises to introduce stable isotope coding of amino acids according to sample origin. This is in effect a type of molecular bar coding that allows a unique mass code to be placed on samples from different sources. It also greatly facilitates comparative quantification studies. After mixing differentially coded samples, it is possible to determine the relative concentration of individual amino acids between these samples in a single analysis. This approach to relative quantification should be of great value in comparative metabolomics.

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