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Technical Notes

Enzymatic Decarboxylation of Tyrosine and Phenylalanine To Enhance Volatility for High-Precision Isotopic Analysis

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We present a rapid and selective method to increase the volatility of tyrosine and phenylalanine without adding derivative C for high-precision gas chromatography−continuous-flow isotope ratio mass spectrometry (GCC−IRMS) based on enzymatic decarboxylation to yield alkylamines and evaluated for ^{15}N isotopic integrity. Purified tyrosine and phenylalanine were converted to tyramine and phenethylamine by tyrosine and phenylalanine decarboxylases, respectively. GC separation was achieved using a thick stationary phase (5-μm) capillary column. Recoveries were 95 ± 2%. The reproducibility of δ^{15}N of tyramine and phenethylamine measured by GCC−IRMS averaged SD(δ^{15}N) = 0.33‰. The absolute differences between δ^{15}N of amino acids measured by elemental analyzer-IRMS and the alkylamines measured by GCC−IRMS was not significant. Phenethylamine and tyramine prepared from a mixture of 18 amino acids were extracted by ethanol with 95% recovery, and analysis yielded clean chromatograms and equivalent precision. These data indicate that enzymatic decarboxylation of phenylalanine and tyrosine is a convenient method to increase their volatility for continuous-flow isotopic analysis without introducing extraneous C or significant isotopic fractionation.

Stable isotope tracer experiments using high-precision isotope ratio mass spectrometry (IRMS) are used extensively in many branches of biological research, including biochemistry, biomedicine, pharmacology, and ecology.[1] Recent applications include the study of amino acid kinetics,[2] assessment of amino acids requirement,[3] measurement of plasma protein synthesis rate in humans,[4,5] and ^{13}C-based breath tests.[6]

Isotopic measurement of ^{15}N at high precision and around natural abundance has wide application in amino acid synthesis and metabolism studies.[7−9] In enriched tracer applications, compound-specific isotopic analysis (CSIA) of ^{15}N abundance by gas chromatography−continuous-flow isotope ratio mass spectrometry (GCC−IRMS) has been used to study the effect of the nonsteroidal antiinflammatory agent ibuprofen on the attenuation of the acute-phase response in colon cancer patients, utilizing ^{15}N glycine as a tracer.[10] ^{15}N-Labeled urea has been used as a tracer to show that *Helicobactor pylori* utilize urea as a source of nitrogen for synthesis of its amino acids.[11]

Three major challenges encountered in high-precision CSIA of ^{15}N isotopic abundance, relative to C, have been outlined previously.[12] First, the concentration of N compared to C in most organic compounds is low. For example, amino acids have 2−11 mol of C/mol of N. Second, for ^{15}N/^{14}N isotopic analysis, organic nitrogen must be reduced to N_2, halving the analysis gas relative to CO_2 used for C and resulting in a CO_2/N_2 ratio twice that of the sample C/N ratio. For the amino acids, the production of 1 mol equiv of N_2 results in the formation of 4−22 mol equiv of C, without taking into consideration the carbon of the derivatizing reagent. Finally, the preponderance of N_2 in ambient air translates

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into high N_2 backgrounds even for small system leaks. These factors effectively increase sample size requirements.

Matthews and Hayes[13] first presented a system on-line separation of individual compounds for C and N isotopic measurements using a single collector instrument and calling their technique isotope ratio-monitoring gas chromatography−mass spectrometry (irmGCMS). Combustion converts most organic N into N_2; the CO_2 of combustion must be trapped at liquid N_2 temperature to avoid co-introduction of CO_2 into the ion source with N_2; electron impact of CO_2 creates CO^+, which is an isobaric interference with N_2 at m/z 28. In 1994, Preston and Slater reported a system with a conversion interface consisting of combustion furnace and liquid N_2 cold trap to capture CO_2 and H_2O[14] using a modern multicollector system. In the same year, Merritt and Hayes presented a similar system with the addition of a reduction furnace to convert residual nitrogen oxides to N_2 for highest precision.[15] Instruments for N-CSIA have been available for several years and are generally based on a combustion/reduction approach.

Amino acids are usually derivatized prior to GC analysis and for continuous-flow ^{15}N isotopic analysis to improve volatility and reduce H-bonding. Normal derivatization adds a number of moles of extraneous carbon to each mole of amino acid, the total number depending on the specific derivative group. Typical amounts of extraneous C are 5 mol of C/mol of amino acid for *N*-acetylisopropyl derivatives (NAP),[16−19] 5 mol of C/mol of amino acid for *N-tert*-butyldimethylsilyl-*N*-methyltrifluoracetamide (MTBSTFA) derivatives,[20] and 8 mol of Cmol of amino acid for *N*-pivaloylisopropyl (NPP).[17] While addition of extra C improves signal for GC-flame ionization detection (FID), it poses problems for high-precision isotopic analysis. CO_2 from derivative C cannot be distinguished from CO_2 from the analyte; thus standards and correction factors must be applied for C analysis, as outlined previously.[21] For N analysis, high CO_2/N_2 prematurely occludes cryotraps and degrades chromatographic performance. Ideally, the volatilization chemistry should introduce no extraneous C.

We recently presented a general strategy for volatilization of nonpolar amino acids by chemical reduction with $NaBH_4$ to yield amino alcohols.[21] While this approach is potentially applicable to many more amino acids, the reactivity of chemical reagents produces complex product mixtures, often necessitating time-consuming purification before or after the reaction. In contrast, enzyme-mediated reactions are highly specific for analyte or reaction and are usually less prone to catalyze side reactions. Enzymes have not been exploited as catalysts to alter analytes for high-precision isotopic analysis.

In this paper, we demonstrate a strategy for increasing the volatility of tyrosine and phenylalanine by enzymatic decarboxylation to tyramine and phenethylamine. We present methods for decarboxylation and extraction of tyramine and phenethylamine from a mixture of 18 amino acids, show a GC method suitable for ^{15}N isotopic analysis by continuous-flow GCC−IRMS, and evaluate possible isotopic fractionation by measuring the $\delta^{15}N$ of amino acids by elemental analyzer (EA)-IRMS and their corresponding decarboxylated compounds.

EXPERIMENTAL SECTION

Amino Acids, Solvent, and Reagents. Purified and mixed amino acids were analytical grade, >98% purity, and obtained from Sigma Chemical Co. (St. Louis, MO). Sodium acetate, acetic acid, and ammonium hydroxide were obtained from Fisher Scientific Co. (Fair Lawn, NJ). Tyrosine decarboxylase and phenylalanine decarboxylase are derived from the bacteria *Streptococcus faecalis*, and their concentration is expressed in activity units, where 1 unit decarboxylates 1 μmol of tyrosine or phenylalanine per minute. Tyrosine decarboxylase (0.05 unit/mg of solid with excess pyridoxal 5-phosphate) and phenylalanine decarboxylase enzymes (from *S. faecalis*, 5 units/g) were obtained from Sigma Chemical Co.

Decarboxylation of Tyrosine and Phenylalanine Standards. For evaluation of isotopic integrity by bulk analysis, purified tyrosine and phenylalanine standards were decarboxylated in separate experiments. Relatively large amounts were used because of the sample size requirements of bulk isotopic analysis, ~1 mg of amino acid per determination, as detailed below. Tyrosine and phenylalanine were reacted according to procedures described previously[22] with minor modifications. Tyrosine (100 mg) was dissolved in 50 mL of water with addition of 1 drop of 5 N NaOH to assist in solubilization. A 0.5-mL aliquot of this solution, 2 mg of tyrosine/mL (5.5 μM), was transferred to a small vial and the solvent was evaporated under nitrogen.

Tyrosine decarboxylase (0.275 mg, equivalent to 0.01 unit), suspended in 0.1 M acetate buffer (2 mL, pH 5.5), was added and the mixture was shaken gently at 37 °C. After 3 h, the mixture was boiled to deactivate the enzyme and ultrafiltered (3000 MW cutoff) to remove protein; solvent was removed by evaporation. The sample was dissolved in 0.2 mL of water for analysis.

Phenylalanine was decarboxylated according to procedures described previously[22] with minor modifications. Phenylalanine (100 mg) was dissolved in 25 mL of water; 0.25 mL of the solution, equivalent to 1 mg (6 μmol), was transferred to a small vial, and the solvent was evaporated under nitrogen. Enzyme powder (165 mg), equivalent to 0.8 unit of phenylalanine decarboxylase, suspended in 0.1 M acetate buffer (2 mL, pH 5.5) was added to the vial, and the mixture was shaken gently at 37 °C for 3 h. The mixture was then boiled, utrafiltered, evaporated, and finally taken up in 0.2 mL of water.

Decarboxylation of Phenylalanine and Tyrosine in a Mixture of 18 Amino Acids. A mixture of 18 amino acids in HCl (0.1 M, 1 mL, 2.5 μmol of each amino acid) was dried under nitrogen. Tyrosine decarboxylase and phenylalanine decarboxylase (2.5 units each) suspended in 0.1 M acetate buffer (2 mL, pH 5.5) at 37 °C were added to the mixture, which was then shaken gently for 3 h. Again the mixture was boiled, utrafiltered, and evaporated to dryness. The dried residue was extracted with 500 μL of 95% ethanol. The ethanol was evaporated, and

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the residue was taken up in water as a solvent for introduction to the GC.

Yield and Purity of Tyramine and Phenethylamine. Aqueous solutions of tyramine and phenethylamine were injected into a HP 5890 GC equipped with a CP-sil 5CB fused-silica capillary column (50 m × 0.53 mm × 5 μm film thickness; Chrompack Inc.) with He carrier gas flow of 3.3 mL/min. Injection of 1 μL was performed using split mode 1:20. The oven temperature was isothermal at 250 °C. The injector and detector temperatures were 240 and 250 °C, respectively, for all GC analyses. Detection was by FID. Standard solutions of tyramine and phenethylamine were prepared to permit evaluation of GC-FID response, to allow calculation of yield.

Preparation of Samples for Isotopic Analysis. An EA coupled to an IRMS was used to determine δ^{15}N for nonvolatile tyrosine and phenylalanine standards. Histamine was used as a convenient isotopic standard because it is amenable to direct isotopic analysis by GCC−IRMS and EA-IRMS. Concentrated stock solutions were prepared for histamine, tyrosine, and phenylalanine to ensure isotopic homogeneity.[23] From these solutions, samples of histamine, phenylalanine, and tyrosine containing 80 μg of total N for each were prepared for isotopic analysis in triplicate. A 20 μg/μL standard aqueous solution of histamine was transferred (10.6 μL) to tin capsules. For tyrosine, 520 μL of a 100 μg/50 μL standard solution in water was used, and for phenylalanine, 236 μL of 100 μg/25 μL aqueous solution was used. Water solvent was evaporated to dryness under nitrogen prior to EA analysis. Elemental analysis was performed using a Europa Scientific (Crewe, U.K.) ANCA elemental analyzer coupled to a model 20/20 IRMS.

GCC−IRMS. The on-line isotopic analysis system consisted of a Varian GC (3400) coupled to a Europa Scientific 20/20 IRMS via an in-house-built combustion reactor/reduction reactor/water trap/liquid N_2 cryogenic trap/open split interface. The GC was equipped with CP-sil 5CB fused-silica capillary column identical to that used for GC-FID analysis, as well as its own FID. By means of an automated two-position valve (Valco Instruments, Houston, TX) the analyte could be directed either to the FID for quantification and method development or to IRMS for isotopic analysis. The oven temperature was isothermal at 250 °C, and the injector and the detector temperatures were as before.

The combustion furnace was made of 20 cm × 0.25 mm of deactivated fused silica filled with oxidized Cu wire surrounded by a heated ceramic furnace held at 850 °C. The reduction reactor was made of 20 cm × 0.25 mm deactivated fused silica filled with Cu wire surrounded by a heated ceramic furnace that was held at 550 °C. The water trap was of the Nafion type (DuPont, Wilmington, DE), which eliminates H_2O while retaining CO_2. The cryogenic trap consisted of a stainless steel capillary (30 cm × 1 mm i.d.), which passes through a Dewar filled with liquid nitrogen, and is connected upstream to the water trap exit and downstream to the open split. Separated analyte from the GC is swept into the combustion furnace by He carrier gas and quantitatively combusted into CO_2, H_2O, N_2, and N_xO_y. The products pass into a reduction furnace, where N_xO_y are reduced to N_2.

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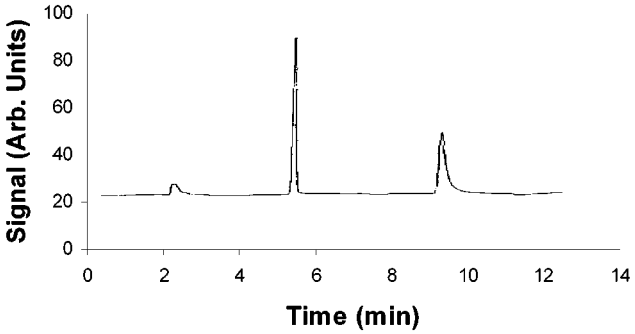

Figure 1. Chromatography of the extracted phenethylamine and tyramine from a mixture of 18 amino acids on CP-sil 5 CB fused-silica capillary column (50 m × 0.53 mm; 5-μm film thickness) (Chrompack Inc.) with He carrier gas. The oven temperature was held at 250 °C.

One-microliter splitless injections were made of 2 μg/μL solutions of phenethylamine (16.6 nmol) and tyramine (14.5 nmol), containing 233 and 212 pmol of N_2, respectively. About 7.7% of the analysis stream is admitted to the IRMS with the rest vented to atmosphere via the open split, representing a ∼13:1 split.

RESULTS AND DISCUSSION

Decarboxylation and Chromatography. Alkylamines that are produced by decarboxylation of the amino acids are strongly H-bonding analytes, which tend to interact with the walls of the fused-silica capillary column when analyzed with a conventional 0.25-μm film capillary column. To avoid tailing, we used the same strategy as reported previously[21] to analyze amino alcohols. A thick stationary phase (5 μm) masks analyte from the capillary column walls when analyte loads well below column capacity are injected.

Decarboxylation of purified phenylalanine or tyrosine produces clean chromatograms with no evidence of any contaminants (data not shown). Comparison of peak areas with standards demonstrates a yield of 95 ± 2% for these 3-h reaction times. Figure 1 shows the chromatogram of the decarboxylated products phenethylamine and tyramine, isolated from a mixture of 18 amino acids after enzymatic decarboxylation. Sharp peaks are obtained with no evidence of extraneous peaks reflective of stray reagents or unreacted materials. Chromatograms such these are compatible with GC introduction to IRMS for isotopic analysis and are ideal for quantitative analysis as well. Yields from the mixture were 95 ± 2%, as for the purified standards.

The results of the nitrogen isotopic analyses of the alkylamines measured by GCC−IRMS and of the amino acids measured by EA-IRMS are presented in Table 1. Tyrosine and phenylalanine averaged $\delta^{15}N_{air} = 8.02‰$ and 1.03‰, respectively, while tyramine and phenethylamine averaged $\delta^{15}N_{air} = 8.28‰$ and 1.49‰, respectively. The analytical error obtained from replicates of amino acids analyzed by EA-IRMS averaged SD(δ^{15}N) = 0.35‰, while the average error of the alkylamine replicates measured by GCC−IRMS was SD(δ^{15}N)= 0.31‰.

The difference between δ^{15}N of the EA analysis of the amino acid and δ^{15}N of the GCC−IRMS analysis of the corresponding alkylamines shown in Table 1 averaged $\Delta\delta^{15}N = -0.36‰$, and they were not statistically significant ($p < 0.05$, Student's *t*-test).

Table 1. Atom Percent (AP) ^{15}N (and δ ^{15}N (‰)) of Tyramine and Phenethylamine Measured by EA-IRMS and GCC−IRMS

	amino acid[a]		alkylamine[b]		deviation	
	AP	$\delta^{15}N$ (‰)[c]	AP	δ ^{15}N (‰)	$AP_{EA}-AP_{GC}$	$\Delta\delta^{15}N$ (‰)[d]
tyrosine/tyramine			0.369 49	8.72		
	0.369 22	8.00	0.369 21	7.96		
	0.369 15	7.80	0.369 38	8.43		
	0.369 32	8.26	0.369 23	8.01		
	0.369 23 ± 0.000 09[e]	8.02 ± 0.23	0.369 32 ± 0.000 13	8.28 ± 0.36	−0.000 09	−0.26
phenylalanine/phenethylamine			0.366 92	1.70		
	0.366 52	0.6	0.366 87	1.55		
	0.366 85	1.49	0.366 71	1.12		
	0.366 67	1.01	0.366 88	1.57		
	0.366 68 ± 0.000 17	1.03 ± 0.45	0.366 85 ± 0.000 09	1.49 ± 0.25	−0.000 17	−0.43

[a] Analysis by EA-IRMS. [b] Analysis by GCC−IRMS. [c] Units are $\delta^{15}N = [(R_{sample} - R_{air})/R_{air}] \times 1000$, where $R_X = [^{15}N]/[^{14}N]$. [d] $\Delta\delta^{15}N$ determined as the difference between EA analysis of amino acid and GC analysis of the alkylamine. Neither difference is significant by *t*-test ($p < 0.05$). [e] Mean ± SD.

This is consistent with expectations, since N isotope fractionation is limited to residual secondary isotope effects associated with unreacted analyte because the amine group is not involved in bond-breaking/bond-making. Together with the recovery data, these results indicate that fractionation is negligible under our conditions.

CO_2 and H_2O are the major products of phenethylamine and tyramine combustion. CO_2 is cleared by liquid nitrogen trapping, which would otherwise result in ion currents at masses 28, 29, and 30 via its CO^+ fragment and preclude high-precision N_2 analysis. Greater amounts of CO_2 relative to N_2 cause correspondingly shorter lifetime for the cryotrap. Tyrosine and phenylalanine have an N content of 7.7% and 8.5%, respectively, which is the lowest for all codon-coded amino acids. By decarboxylation, the nitrogen content of the tyramine and phenethylamine increases to 10.2% and 11.6%. If tyrosine and phenylalanine were derivatized using, for example, the NAP method, 5 mol of C would be added to every mole of amino acid, decreasing the N content of tyrosine and phenylalanine to 4.9% and 5.2%. In most derivative methods, derivatization of the amine is required, which may induce isotopic fractionation if the reaction is not quantitative. Decarboxylation avoids possible isotopic fractionation since the amino group is not involved in the reaction.

Various strategies have been used to measure the enrichments of the tracer in the plasma. Phenylalanine and tyrosine were separated from the plasma by ion exchange HPLC after being derivatized with *o*-phthalaldehyde and then analyzed by GC quadrupole mass spectrometry.[24] In other studies, amino acids in plasma were converted to their butyldimethylsilyl derivatives and isotopic enrichment was analyzed by GC quadrupole mass spectrometry.[25,26] These amino acids also have been also converted to methylated pentafluorobenzyl derivatives, and enrichment was detected by GC/MS with electron capture chemical ionization.[27] Amino acids also have been converted to their heptafluorobutyryl *n*-propyl esters, and the enrichment was analyzed by GC with selected ion monitoring-negative chemical ionization mass spectrometry.[28] Among the most sensitive methods for phenylalanine tracer analysis is that of Calder et al.[29] Phenylalanine-d_5 was enzymatically decarboxylated and converted to the heptafluorobutyryl derivative, with analysis by electron impact GC/MS. Decarboxylation improved detection limits by eliminating unspecified interferences and yielded a method sensitive to 0.005 APE enrichments, which is competitive with IRMS detection limits under some circumstances. However, this approach does not apply to ^{15}N labeling since the ^{13}C isotopomers would cause a high background and dramatically degrade detection limits and cannot provide high-precision results. The present method is very rapid for $^{15}N/^{14}N$ analysis, in part because of a particularly straightforward recovery procedure due to the specificity of the enzymes and the difference in chemical properties between the product alkylamines and remaining amino acids.

This decarboxylation strategy can be also applied to metabolism studies using ^{13}C tracer compounds that are labeled at positions other than COOH and for ^{2}H tracers using GC/MS analysis. Commercially available alternatives exist for tyrosine and phenylalanine ^{13}C-labeled in a variety of positions. Tracers labeled with ^{13}C in every ring position should be particularly sensitive probes when analyzed by GCC−IRMS. In addition to phenylalanine and tyrosine, decarboxylases are available for at least four other amino acids: arginine, histidine, lysine, and glutamine. The method described here should be readily adaptable to these compounds.

Enzymatic removal of polar/H-bonding groups is a general strategy for increasing analyte volatility for isotopic analysis. Our results suggest that enzymatic deamination may be effective for isotopic analysis of amino acids as well. Deamination is a viable alternative to decarboxylation when $\delta^{15}N$ is not desired or when side group $\delta^{15}N$ are to be measured. At least nine amino acid deaminating enzymes are available commercially, specifi-

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cally L-alanine dehydrogenase, phenylalanine dehydrogenase, asparaginase, lysine oxidase, leucine dehydrogenase, glutaminase, tryptophanase, histaminase, and aspartate amino transferase. Additionally, L-amino acid oxidase produces the α-oxoacid of many amino acids, specifically, leucine, isoleucine, norleucine, α-aminobutyric acid, phenylalanine, tyrosine, tryptophan, norvaline, methionine, histidine, and citrulline, and may apply also to serine, threonine, aspartic acid, glutamic acid, lysine, and ornithine;[30] many of the resulting oxoamino acids may provide sufficiently volatile for GC analysis. D-Amino acid oxidase applies to several D-amino acids as well. Thus, commercially available decarboxylases or deaminating enzymes apply to most amino acids and may prove important for general applications, as well as to the specialized application shown here.

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