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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH THERMOSPRAY MASS SPECTROMETRIC DETECTION OF α-CARBOXYAMIDO AMINO ACIDS[a]

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SUMMARY

A method is described for high-performance liquid chromatographic separation of underivatized α-carboxyamido amino acids, with sensitive detection by thermospray mass spectrometry. The unit mass difference between α-carboxyamido (amidated) amino acids and their free-carboxyl analogs allows mass spectrometric detection of amidated amino acids in the presence of the usual excess of non-amidated amino acids found in samples from biological sources. Separation is carried out on an exhaustively end-capped octadecylsilyl reversed-phase support with an aqueous mobile phase containing 10 m*M* ammonium *p*-toluenesulfonate (pH 3.5) and 0–30% methanol. Using these conditions, the α-amidated amino acids are clearly separated from their analogous free-carboxyl derivatives. Ammonium formate is added post-column to provide the necessary ions for efficient thermospray detection of the eluting amino acids. Detection of the α-amidated amino acids at *ca.* 1 pmol on-column is achieved in the single ion monitoring mode. This method allows unequivocal identification of α-carboxyamido amino acids enzymatically released from amidated peptides, without the necessity for any extraction of derivatization procedures.

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[a] The opinions and assertions contained herein are the private views of the authors and are not to be construed as official or reflecting the views of the Department of the Navy, the Department of Defense or the Department of Health and Human Services.

INTRODUCTION

Many biologically active peptides, including most peptide hormones, contain amino acids which have been post-translationally modified. These modifications are often essential for complete biological activity, and it is recognized that identification of such modifications on a novel peptide suggests a hormonal function for that peptide. The most common post-translational modification of peptides is the occurrence of an α-carboxyamido moiety on the C-terminal amino acid, instead of the usual free α-carboxylic acid, *i.e.* an amidated amino acid. This modification has been identified on approximately half of the known mammalian gastrointestinal and neuroendocrine peptide hormones (*e.g.* gastrin, cholecystokinin, vasopressin and oxytocin) as well as many peptide hormones from other phyla (for instance frog bombesin, scorpion venom peptides, crustacean red pigment concentrating hormone, and insect adipokinetic hormones). Identification of peptides with C-terminal amidated amino acids has been used as a criterion for screening for novel peptide hormones[1], which has led to the discovery of novel biologically active peptides such as neuropeptide Y and pancreastatin.

The original method described for identification of amidated peptides in peptide mixtures involves enzymatic hydrolysis to liberate C-terminal amidated amino acids, unmodified amino acids, and small peptides, followed by derivatization of the mixture to yield N-dansylated derivatives, then a "specific" extraction of the dansyl α-amidated amino acids, and identification by thin-layer chromatography (TLC)[1]. This method produces a complex mixture of amino acid and peptide derivatives, which must be separated before identification of any α-amidated amino acids present. The alkaline/organic extraction method used biases the procedure towards identification of amino acid amides with non-polar side-chains, and the two-dimensional TLC detection is limited in capacity and sensitivity as well as being very labor-intensive. Modifications to the detection method using high-performance liquid chromatographic (HPLC) separation and detection have been reported, using phenylthiocarbamoyl[2,3] or dansyl[4] derivatives, but these still require extraction and derivatization steps. The problems with these methods, including inability to detect amides of polar amino acids and under- and over-derivatization, have been described[5].

The one-unit difference between the masses of an amidated amino acid ($-CONH_2$, identified here by a #) and its free-carboxyl congener ($-COOH$) suggests that mass spectrometry (MS) would be the ideal method for analysis of amino acid amides in the presence of non-amidated amino acids. This approach would obviate the necessity for specific extraction of amino acid amides or specific derivatization procedures. Thermospray MS is suited to identification of low-molecular-weight polar non-volatile molecules such as amino acids[6], and allows coupling to HPLC systems for separation of any compounds of the same mass (examples of such isobaric compounds include isoleucineamide and leucineamide, asparagine and aspartic acid amide, asparagineamide and leucine). Generally, MS techniques reported for amino acids are either not currently amenable to the solvent flows of HPLC (secondary-ion MS[7]), require derivatization to produce chromatographable analogues (gas chromatography–MS), or have high background at low masses and so require derivatization to produce high-mass ions for amino acids (continuous-flow fast atom bombardment MS[8]).

There are many LC and HPLC techniques reported for separation of amino acids[9,10] and amino acid amides[2–4,11]. The majority of these demand pre-column derivatization for adequate chromatography and detection, and/or use solvents containing involatile salts that are not compatible with thermospray MS. We report here a thermospray HPLC–MS method for high sensitivity analysis of amino acid α-carboxyamides that requires no extraction or derivatization procedures.

EXPERIMENTAL

Mass spectrometry

The mass spectrometer used in all these studies was a Hewlett-Packard MSD equipped with a Vestec thermospray interface (Vestec, Houston, TX, U.S.A.). The mass spectrometer was operated in the positive ion mode. Moderate probe tip (215°C), vapor (280–310°C) and block (400°C) temperatures were maintained. Data was accumulated in the single ion monitoring (SIM) mode using low resolution (± 0.45 mass units) with dwell times ranging from 200 to 500 ms/ion. Full-scan spectra were usually accumulated over mass ranges from 70 to 210 mass units, at rates of 2–4 s/scan.

High-performance liquid chromatography

A Pharmacia/LKB Model 2150 pump provided the constant solvent flow necessary for stable background ion current (Pharmacia/LKB, Piscataway, NJ, U.S.A.). A low-pressure solvent mixer and a Pharmacia/LKB Model 2152 controller were used to generate linear gradients in organic modifier content, as required, using the single pump. Mobile phase flow-rates were usually 1.0 ml/min for 4.0 and 4.6 mm (I.D.) columns. Post-column addition of ammonium formate to provide the ammonium concentration needed for efficient thermospray positive ion generation was carried out using a second Pharmacia/LKB 2150 pump and a low-volume tee joint. Post-column solvent flow-rate and concentration were adjusted to provide a final flow-rate into the thermospray source of 1.2–1.25 ml/min and an ammonium ion concentration of 0.1–0.2 *M*.

A variety of HPLC columns has been tested. The results shown here were obtained with a Beckmann 5-μm endcapped octadecylsilyl column (15.0 × 4.0 mm I.D.). Equivalent results have been obtained using a variety of octadecylsilyl columns from Pharmacia/LKB, and polymeric octadecylsilyl columns (Excellopac ODS-A from R. Gourley, Laurel, MD, U.S.A.). Column-to-column differences usually require adjustment of the organic modifier gradient to maintain adequate resolution.

Chemicals and solutions

Standard L-amino acids and L-amino acid amides were obtained from Sigma (St. Louis, MO, U.S.A.) and Bachem (Torrance, CA, U.S.A.). Lysineamide dihydrochloride was a generous gift from Dr. W. H. Simmons, Loyola University Medical Center, Maywood, IL, U.S.A. *p*-Toluenesulfonic acid was analytical-reagent grade (Eastman-Kodak, Rochester, NY, U.S.A.). The standard used, α-amino-*n*-butyric acid, was obtained from Calbiochem (San Diego, CA, U.S.A.). Water used was purified by reverse osmosis and ion exchange. Other chemicals and solvents were analytical grade.

Stock solutions of amino acids and amino acid amides were prepared in filtered

0.1 *M* ammonium acetate or 0.1 *M* ammonium formate (pH 7.0) generally at a concentration of 10 or 20 m*M*. Most solutions were found to be stable at room temperature for extended periods (up to 6 months), although some were made up more frequently, or stored in aliquots at −20°C and thawed as required. Working solutions for determining retention characteristics were diluted daily in mobile phase to a final concentration of 0.1 m*M*. Solutions for signal-to-noise determinations and standard curves ranged in concentration from 20 n*M* to 1.0 m*M*.

Solvents were prepared by dissolving the mobile phase modifier (*p*-toluenesulfonic acid, 10 m*M*) in water or water–methanol and adjusting to the desired pH with concentrated ammonium hydroxide. The solutions were then filtered (0.45 μm filter) and degassed under vacuum immediately prior to use. Stock solutions of 1.0 *M* ammonium formate were prepared and were diluted as required with water and filtered (0.45 μm) and degassed prior to use.

RESULTS

The one- and three-letter abbreviations used in the text and figures for amino acids and amino acid amides, the mass-to-charge ratio (*m/z*) of the ions used for detection and quantification, and the retention times under the conditions described in the legend to Fig. 1, are presented in Table I.

In all cases the ion used for quantification is that of the protonated monocationic molecular ion $[M+H]^+$. Most amino acids and amino acid amides had simple spectra dominated by the protonated molecular ion, although some spectra showed significant fragment ions (*e.g.* *m/z* 130 for $[Glu+H - H_2O]^+$). Arg and Arg# had complex

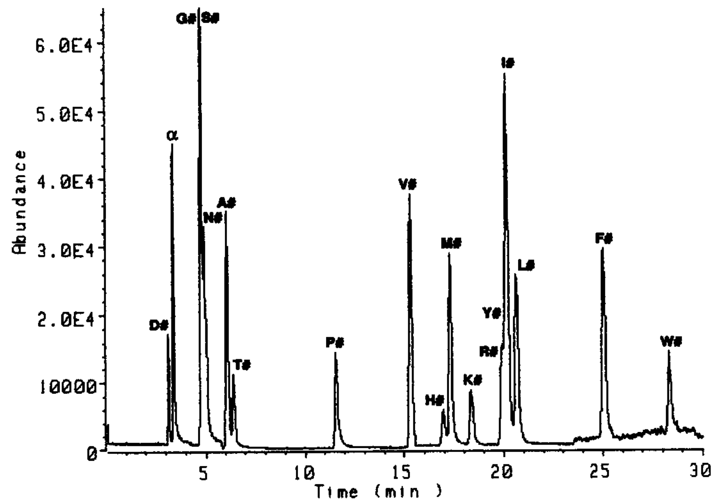

Fig. 1. Summated SIM profiles for thermospray HPLC–MS of a mixture of seventeen α-carboxyamido amino acids and α-amino-*n*-butyric acid. A 20-μl volume of a solution containing 0.1 m*M* of each compound in mobile phase was injected at 1.0 min. The mobile phase contained 10 m*M* *p*-toluenesulfonic acid, pH adjusted to 3.5 with ammonium hydroxide, and a linear gradient of methanol from 0 to 30% at 1.5%/min beginning at 5.0 min. The flow-rate through the 5-μm endcapped octadecylsilyl column (15 cm × 4.6 mm I.D.) was 1.0 ml/min, and 0.5 *M* ammonium formate at a rate of 0.25 ml/min was added post-column.

TABLE I

ABBREVIATION CODES, PROTONATED MOLECULAR ION MASSES, AND CHROMATOGRAPHIC RETENTION TIMES FOR α-AMINO-*n*-BUTYRIC ACID, THE TWENTY COMMON PROTEIC AMINO ACIDS AND SEVENTEEN AMINO ACID α-CARBOXYAMIDES

The α-carboxyamides of cysteine, glutamic acid, and glutamine were not available. α-Amino-*n*-butyric acid was used as a standard only; its α-carboxyamide is not expected to be found in any mammalian peptide.

"Parent" amino acid	*Three-letter abbrev.*[a]	*One-letter abbrev.*[a]	*Amide*		*Acid*	
			$[M+H]^+$	t_R^b	$[M+H]^+$	t_R^b
Alanine	Ala	A	89	5.04	90	1.80
Cysteine	Cys	C	121	–	122	1.60
Aspartic acid	Asp	D	133	2.11	134	1.47
Glutamic acid	Glu	E	147	–	148	1.76
Phenylalanine	Phe	F	165	24.05	166	17.15
Glycine	Gly	G	75	3.75	76	1.59
Histidine	His	H	155	17.35	156	6.08
Isoleucine	Ile	I	131	19.18	132	12.73
Lysine	Lys	K	146	15.94	147	4.68
Leucine	Leu	L	131	19.63	132	13.28
Methionine	Met	M	149	16.30	150	6.03
Asparagine	Asn	N	132	3.99	133	1.59
Proline	Pro	P	115	10.56	116	2.16
Glutamine	Gln	Q	146	–	147	1.90
Arginine	Arg	R	174	18.92	175	10.79
Serine	Ser	S	105	3.89	106	1.59
Threonine	Thr	T	119	5.38	120	1.76
Valine	Val	V	117	14.33	118	5.36
Tryptophan	Trp	W	204	27.33	205	23.90
Tyrosine	Tyr	Y	181	19.10	182	13.71
α-Amino-*n*-butyric acid	Aab[c]	α[c]	–	–	104	2.40

[a] The symbol used in this article to denote an α-carboxyamido amino acid is, *i.e.* asparagineamide is abbreviated to Arg# or R#.

[b] The retention time tabulated is minutes after injection, which was at 1.0 min after commencement of the run.

[c] There is no commonly accepted three- or one-letter abbreviation for α-amino-*n*-butyric acid. Aab and α are used in this text.

spectra, which showed changes that could be correlated with the cleanliness of the thermospray probe: a new probe gives spectra for Arg in which the base peak has *m/z* 115, while older probes produce *m/z* 157 (assigned to $[\text{Arg}+\text{H} - \text{H}_2\text{O}]^+$) or the molecular ion (*m/z* 175) as the base peak. This change is probably due to coating of reactive sites with a carbonized layer of organic material as the probe is used. Similar spectral changes can be deliberately induced by using non-reactive glass coatings inside the stainless-steel thermospray probe[12].

Fig. 1 shows a chromatogram of a mixture of the seventeen available amidated amino acids and the standard α-amino-*n*-butyric acid. The chromatogram has the appearance of a normal liquid chromatogram, but is actually a summation of a number of individual ion profiles. This is the reason for the sudden changes in baseline intensity, for example at 23.5 minutes. The SIM data acquisition parameters were such that a set of two to four ions was acquired concurrently, with the start and end times of each group chosen from previous retention time determinations. The mobile phase pH and organic modifier gradient were designed to separate isobaric amidated amino acids (Ile#, Leu#) from each other, and also amidated amino acids from any isobaric amino acids (*e.g.* Asp#, Asn). Some of the amino acid amides are not totally chromatographically resolved, however the SIM profiles of the molecular ions are resolved as the overlapping peaks are not isobaric. Fig. 2 demonstrates the resolution of the individual SIM profiles for the group of amidated amino acids eluting at *ca.* 5 min after injection.

Fig. 3a shows the chromatographic profile of the set of twenty common proteic amino acids and α-amino-*n*-butyric acid under identical conditions to those shown for Fig. 1. Resolution is poor for some of the early-eluting amino acids as the chromatographic conditions were optimized for separation of the amino acid amides, as described above. The SIM parameters used for this sample are not the same as those used for detection of the amidated amino acids in Fig. 1. When the mixture of twenty amino acids and Aab was chromatographed using the SIM parameters optimized for the amidated amino acids, only three compounds were detected (Fig. 3b). These compounds are the internal standard Aab, which is present in both mixtures, and compounds having masses equivalent to Asp# and Pro#. Upon inspection of the actual retention times, the amino acids responsible for these peaks were identified as Asn and Arg, respectively. Asn and Asp# are isobaric, and Pro# is isobaric with the *m/z* 115 fragment ion of Arg described above. As shown in Fig. 3c and 3d, which are "close-up" views of the relevant sections of Figs. 1 and 3a, in both cases the interfering amino acid is resolved from the amino acid amide.

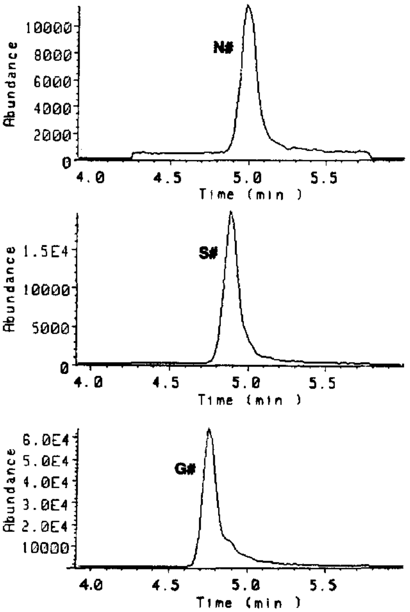

Fig. 2. Individual SIM profiles for three closely eluting amino acids amides from Fig. 1. These profiles are taken from the same data as that shown in Fig. 1, but are presented as individual profiles on an expanded scale to demonstrate the resolution of closely eluting non-isobaric compounds.

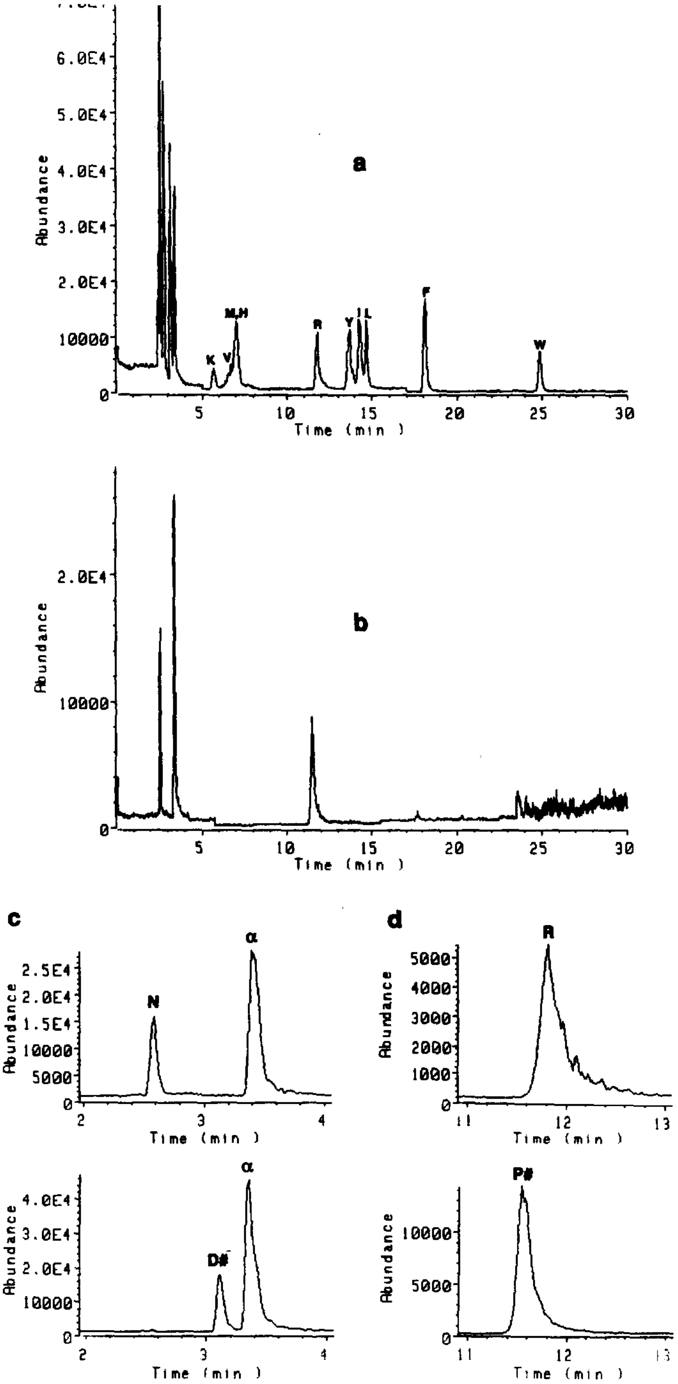

Fig. 3. Thermospray HPLC–MS profiles of amino acids. (a) Summated SIM profiles for a mixture of the twenty common amino acids and α-amino-*n*-butyric acid, chromatographed under the same conditions described in Fig. 1. As described in the text, the SIM parameters used for this sample were those optimized for the amino acids. The early-eluting group of amino acids comprised D, S, N, G, C, E, T, A, Q, P and $^{\alpha}$. (b) Summated SIM profiles for the same mixture of amino acids, chromatographed under the same conditions, but with the SIM parameters optimized for the detection of amino acid amides (*i.e.* the SIM parameters used for Fig. 1). Only two of the twenty non-amidated amino acids appear as amidated amino acids. (c) and (d) Demonstration that the amino acids seen using the SIM parameters for amidated amino acids do not co-chromatograph with the authentic amino acid amides. c shows summated SIM profiles for the relevant time period of b (upper) and Fig. 1 (lower), and d shows *m*/*z* 115 SIM profiles from b (upper) and Fig. 1 (lower).

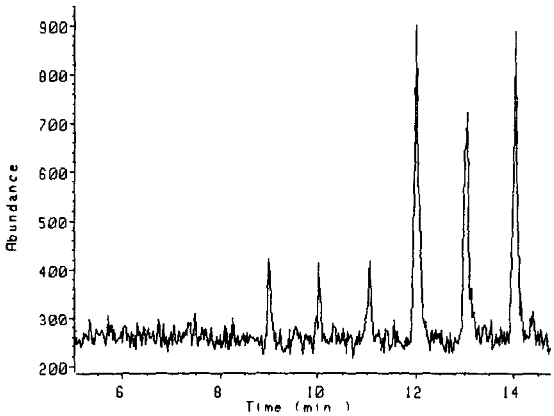

Fig. 4. Detection limit for thermospray HPLC–MS analysis of amino acids amides. SIM profile of *m/z* 89 for triplicate injections of mobile phase, 1 pmol of Ala$^{\#}$, and 4 pmol of Ala$^{\#}$. Injections were made onto the column used for Figs. 1–3, with the starting buffer only: 1.0 ml/min of 10 m*M* *p*-toluenesulfonic acid, pH 3.5 with ammonium hydroxide, no organic modifier, with 0.25 ml/min of 0.5 *M* ammonium formate added post-column. The retention time of Ala$^{\#}$ is 5 min under these conditions, and injections were timed for elution at 6, 7 and 8 min (blank), 9, 10 and 11 min (1 pmol) and 12, 13 and 14 min (4 pmol).

As can be seen from Fig. 1, the peak-height response varies for individual amidated amino acids. The intensity of the molecular ion was not affected significantly by changes in the pH of the post-column buffer, suggesting that the variation is not primarily due to different proton affinities. To some degree the variation can be ascribed to the degree of fragmentation of the protonated molecular ion: the basic amino acid derivatives (Arg#, Lys# and His#), which have the most complex spectra, also have the lowest intensity for the parent molecular ion, while the simplest amidated amino acid, Gly#, has the highest intensity. The MS background is also not equal for all masses (*e.g.* note the noisy baseline from 23.5 to 30.0 min in Fig. 1 due to the ion *m/z* 204). The detection limits therefore vary noticeably for the different amidated amino acids. Fig. 4 shows that for a "typical" amidated amino acid, Ala#, 1 pmol on column is readily detectable, with a signal-to-noise ratio of *ca.* 2:1. As has been previously reported, amino acids and amidated amino acids (*e.g.* released by carboxypeptidase cleavage of amidated peptides) can be quantified over the picomole to nanomole range using an added internal standard, α-amino-*n*-butyric acid[11].

DISCUSSION

The method described here was developed for identification of α-carboxyamido amino acids released by enzymatic digestion of peptide mixtures. Amidated amino acids can be released from peptides by cleavage with a number of carboxypeptidase enzymes[13,14], providing a method for identification of potentially biologically active peptide hormones via detection of post-translationally modified amino acids[1,5]. We have previously presented a preliminary report describing a thermospray HPLC–MS method for analysis of amino acids and amino acid amides[11]. This paper details much-improved method utilizing endcapped octadecylsilyl HPLC columns and a low pH (pH 3.5) buffer containing *p*-toluenesulfonic acid.

Previous methods described for identification of α-carboxyamido amino acids in peptide digestion mixes have involved complicated extraction and derivatization steps[1–4]. The variation in chemical characteristics of the side-chains of the common amino acids makes it unlikely that any extraction–derivatization procedures could be specific for all possible amidated amino acids in the presence of unmodified amino acids and partially cleaved peptides. The use of a mass selective detector allows identification of α-carboxyamido amino acids in the presence of α-carboxyl amino acids due to the one-mass-unit difference between the modified and unmodified analogs. The thermospray HPLC–MS method is generally at least as sensitive as previously reported HPLC methods for amidated amino acid detection, although the sensitivity of detection for individual compounds does vary with the chemical nature of the amino acid amide and the level of background noise at each mass. However, as the thermospray HPLC–MS method requires no extraction or derivatization for detection of the α-carboxyamido amino acids, enzymatic hydrolysates of peptides can be injected directly onto the column, thereby providing a much higher overall sensitivity for detection of α-carboxyamido amino acids from hydrolyses of biological samples.

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