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SENSITIVE AMINO ACID ANALYSIS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

OPTIMIZATION OF THE *o*-PHTHALALDEHYDE METHOD FOR COMPOSITION OF PICOMOLE AMOUNTS OF ACID HYDROLYZATES

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SUMMARY

The *o*-phthalaldehyde method for pre-column derivatization of amino acids for compositional analysis has been optimized for quantitation at the low picomole level. As little as 20 pmol of peptide are hydrolyzed and 6 pmol can be derivatized for analysis. The method results in quantitative recovery of the amino acids and gives reliable quantitation even at the 6 pmol level.

The study details the procedures involved in hydrolyzing small amounts of peptides. The effects of purity and stability of the *o*-phthalaldehyde reagent, pH, and composition of the mobile phase are discussed with a view to adaptation for use with various high-performance liquid chromatographic systems. Optimization of the derivatization conditions and strategies for lengthening column life are described. The relative performances of different reversed-phase columns from two different manufacturers are compared.

INTRODUCTION

o-Phthalaldehyde (OPA) was first shown by Roth[1] to react with primary amines in the presence of β-mercaptoethanol (β-ME) to produce intensely fluorescent compounds. The structure of the fluorescent product in the reaction between the OPA–β-ME adduct and the primary amine was shown to be a 1-alkylthio-2-alkyl-substituted isoindole by Simons and Johnson[2]. The structure of this isoindole explained why OPA does not react with secondary amines. Several post-column derivatization methods for the detection and quantitation of amino acids are currently in use[3–6]. These involve specialized, dedicated instrumentation where the amino acids are separated by ion-exchange chromatography followed by post-column reaction systems. The post-column tubing adds to the dead volume, increasing peak broadening and lowering the sensitivity. Other methods of amino acid analysis involving precolumn derivatization of the amino acids as their dansyl[7,8], phenylthiohydantoin[9] or phenylisothiocyanate[10] derivatives provide less sensitivity and specificity than the OPA–β-ME reagent. Precolumn derivatization with OPA–β-ME and reversed-phase

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high-performance liquid chromatography (HPLC) have been shown to provide a rapid and sensitive method for amino acid analysis[11–17]. However, the sensitivity of this method has not been fully exploited. This paper discusses in detail procedures for obtaining the amino acid composition of as little as 20 pmol of a peptide. It also describes a study on the effect of pH of the mobile phase on the resolution of the various amino acids. Thus any investigator with access to HPLC equipment can utilize this sensitive method by merely adjusting the gradient according to the instrumentation available. In addition, the performance and behavior of two different columns, the Perkin-Elmer 3x3™ column and the Axxichrom 5 μm column, are compared.

MATERIALS AND METHODS

Apparatus

The chromatographic system consisted of two Beckman-Altex Model 110A HPLC pumps (Beckman, Palo Alto, CA, U.S.A.) equipped with an Eldex pressure monitor (Instru-Spec Assoc., Concord, CA, U.S.A.), a Rheodyne injection valve (Model 7125) with a 200-μl loop and an Axxiom 710 HPLC gradient controller (Cole Scientific, Calabasas, CA, U.S.A.). A Shimadzu RF 530 fluorescence HPLC detector (Instru-Spec Assoc.), equipped with a xenon lamp was used to monitor the fluorescence. The fluorometer was set at an excitation wavelength of 340 nm and an emission wavelength of 455 nm. Two different types of column were used: Perkin-Elmer 3x3, ODS, 3 μm, 3 cm × 4.1 mm I.D. (Perkin-Elmer, Norwalk, CT, U.S.A.) and Axxichrom ODS, 5 μm, 15 cm × 4.1 mm I.D. (Cole Scientific). Guard columns were not used, but a column inlet filter with a 2-μm frit (Alltech Assoc., Deerfield, IL, U.S.A.) was placed between the injector and the column. Chromatographic peaks were integrated with an IBM computer. Solvent A consisted of a sodium acetate buffer at various pH values, tetrahydrofuran, and, in some cases, acetonitrile. Solvent B was methanol.

Solvents, reagents and standards

Tetrahydrofuran and methanol were Baker-analyzed, HPLC-grade solvents (obtained from VWR Scientific, Sunnyvale, CA, U.S.A.). Acetonitrile was glass-distilled, low-UV, HPLC-grade (obtained from Alltech Assoc., Los Altos, CA, U.S.A.). The solvents were used without further purification. Water was distilled and deionized through Millipore Milli-Q filters. Amino acid standards, fluoraldehyde, β-mercaptoethanol, methanol for dissolving the OPA, fluoraldehyde solution, Brij® 35 and fluoraldehyde reagent diluent (1 *M* potassium borate, pH 9.5) were purchased from Pierce, (Rockford, IL, U.S.A.). Individual amino acids were obtained from Sigma (St. Louis, MO, U.S.A.).

Preparation of sodium acetate buffers

Sodium acetate (2 *M*, pH 5.4) was prepared by titrating 2 *M* acetic acid with sodium hydroxide pellets to pH 5.4. This solution was diluted to 25 m*M* for use in the mobile phase. Sodium acetate (pH 6.6, 0.5 *M*) was made by titrating glacial acetic acid (diluted in water to make an 0.5 *M* solution) with solid sodium hydroxide pellets to pH 6.6. For pH studies, glacial acetic acid was added to this stock solution to lower the pH. The change in molarity of the acetate was noted. The pH of the diluted solutions was not measured.

Sodium phosphate (1.3 *M*, pH 3.5) was prepared by titrating 1.3 *M* phosphoric acid with sodium hydroxide pellets.

Preparation of amino acid standards

The Pierce amino acid standard H was diluted with 0.1 *N* hydrochloric acid, prepared by diluting Pierce 6 *N* hydrochloric acid used for hydrolysis in distilled, deionized water. The individual amino acids were dissolved in 0.1 *N* hydrochloric acid.

Preparation of the OPA–β-ME reagent

A stock solution of OPA was prepared by dissolving 10 mg of fluoraldehyde in 200 μl of methanol. The dilution buffer for the OPA consisted of 4.425 ml of 1.0 *M* potassium borate (fluoraldehyde reagent diluent), 4.425 ml of water, 30 μl of Brij 35, and 20 μl of β-ME. To this buffer 100 μl of the OPA stock solution were added. Aliquots of the reagent were transferred to several 1.5-ml Eppendorf tubes, which were sealed immediately and refrigerated. A fresh tube was used every day. The stock solution is generally usable for a week. In some cases, a contaminant peak, which appeared to be an oxidation product of OPA, appeared within a week. In this case, the remainder of the OPA batch was discarded and a fresh solution prepared.

Derivatization procedures

Standards or samples (2–25 μl) were mixed with 50 μl of the OPA–β-ME reagent. After 2 min of reaction at room temperature, 25 μl of 1.3 *M* sodium phosphate (pH 3.5) were added and the mixture was allowed to stand for another 30 s before the entire sample was injected into the HPLC system.

Acid hydrolysis of peptides

Peptides were dried in 6 × 50 mm borosilicate tubes, which had been flame-treated by heating to a glowing yellow color. These tubes were then placed in a Pico-Tag (Waters Assoc., South San Francisco, CA, U.S.A.) hydrolysis vessel or vial. Then 200 μl of 6 *N* hydrochloric acid, containing 1 μl of β-ME, were added to the hydrolysis vessel. The hydrolysis vessel was flushed with nitrogen and sealed after evacuation, using the Pico-Tag hydrolysis system. Hydrolysis was carried out at 110°C for 24–26 h. Amino acid standard H was also hydrolyzed along with the peptides in the same hydrolysis vessel or vial. In order to detect cystine and cysteine, the standards or peptides were dried in 6 × 50 mm flame-treated borosilicate tubes, then 10 μl of performic acid solution were added, and the reaction mixture was kept on ice for 2.5–3 h. (The performic acid was freshly prepared from a 10% solution of 30% hydrogen peroxide in formic acid by allowing the mixture to react at room temperature for 2 h.) The performic acid was removed by evaporation, and the contents of the tubes were subjected to acid hydrolysis, as described above.

Following hydrolysis, the hydrolyzates were dissolved in water to bring the final concentration to *ca.* 0.5 pmol/μl of sample or standard. The samples were then transferred to Eppendorf vials with drawn-out Pasteur pipettes and frozen. Samples thus stored are stable for several days. Prior to the addition of water to the hydrolyzates, the maximum volume of water to be added to the hydrolyzates was derivatized with the OPA solution and subjected to chromatography to ensure that the water was free of OPA-positive impurities.

For the hydrolysis of synthetic porcine somatostatin (Sigma), 20 pmol (calculated according to weight of the peptide) were hydrolyzed and dissolved in 40 μl of water; 20 pmol per amino acid of the hydrolyzed standard amino acid mixture were also dissolved in 40 μl of water, and 10–12 μl of this (5–6 pmoles of peptide) were derivatized with the OPA–β-ME reagent. The entire mixture was injected into the HPLC system.

RESULTS AND DISCUSSION

Optimization of the HPLC separation

Effect of mobile phase composition

Effect of acetonitrile. Acetonitrile in buffer A did not significantly affect resolution and was omitted in the later stages of development.

Effect of tetrahydrofuran (THF). THF was found to be necessary for the resolution of glycine from threonine. The separation of the glycine–threonine pair depended more on the THF concentration than on the pH of the mobile phases within the pH range tested. The addition of 2% THF in buffer A gave sharp peaks with good separation of glycine and threonine. However, at 2% THF, it became difficult to separate cysteic acid (CA) from aspartic acid. When the THF concentration in solvent A was 1%, the CA–Asp pair was resolved and the resolution improved with decreasing pH. The carboxylic acid moiety of the Asp is less ionized at lower pH and, hence, it is retained longer on the reversed-phase HPLC column. However, with 1% THF, the peaks were slightly broader.

Effect of ionic strength. The amino acids were retained slightly longer with 50 m*M* sodium acetate in mobile phase A than with 25 m*M*. This has been observed by others[13,17].

Effect of methanol. The effect of methanol (solvent B) was more pronounced in the separation of the peaks later eluted. Steps gradients were more effective than linear gradients in obtaining complete resolution[14].

Effect of pH. The effect of pH is summarized in Table I. It is interesting to note the position of histidine in the two columns relative to the Gly–Thr pair (Fig. 1). As the PE 3x3 column was used longer, the separation of His from the Gly–Thr pair was lost. The Axxichrom column was better for resolution of His as well as Met–Val. From Table I, it can be seen that the best results were obtained by using the Axxichrom column at pH 5.6. These conditions were used for further chromatographic experiments.

In the pH range tested, the resolution of the CA–Asp pair and the Met–Val pair increased with decreasing pH, while the resolution of the Glu–Ser pair decreased with decreasing pH.

Extending the life of the column

The PE 3 × 3 column is useful for rapid analyses, but, in the author's experience, it was not as reliable as the Axxichrom column. In addition, the PE 3x3 column appeared to be much more susceptible to deterioration by repeated injections of the OPA–β-ME–amino acid adduct. The alkaline reaction mixtures are harmful to the silica-based columns and, therefore, the pH must be reduced before injection. Jones *et al.* recommend the use of 0.1 *M* potassium phosphate (pH 4)[15] or

TABLE I

THE EFFECT OF pH ON THE SEPARATION OF AMINO ACID PAIRS

Note that the Axxichrom column at pH 5.6 gave the best results. The term "yes" refers to good separation. The areas under the peaks were quantifiable accurately and reproducibly. The term "no" refers to complete loss of resolution and the term "poor" refers to peaks that were resolved poorly where the areas could not be reliably quantified.

pH	*Column*	*Separation of*				
		His–Gly–Thr	*CA–Asp*	*Ala–Arg*	*Met–Val*	*Glu–Ser*
5.4	PE-3x3	No		Yes	Yes	No
5.4	Axxichrom	Yes		Yes	Yes	No
5.6	PE-3x3	No	Poor	Yes	Yes	Yes
5.6	Axxichrom	Yes	Yes	Yes	Yes	Yes
5.75	PE-3x3	No			Yes	Yes
5.75	Axxichrom	Yes			Yes	Yes
6.0	PE-3x3	Yes		No	Yes	Yes
6.0	Axxichrom	Yes		No	Yes	Yes
6.6	PE-3X3	No	No	Yes	No	Yes
6.6	Axxichrom	Yes	Poor	Yes	Yes	Yes

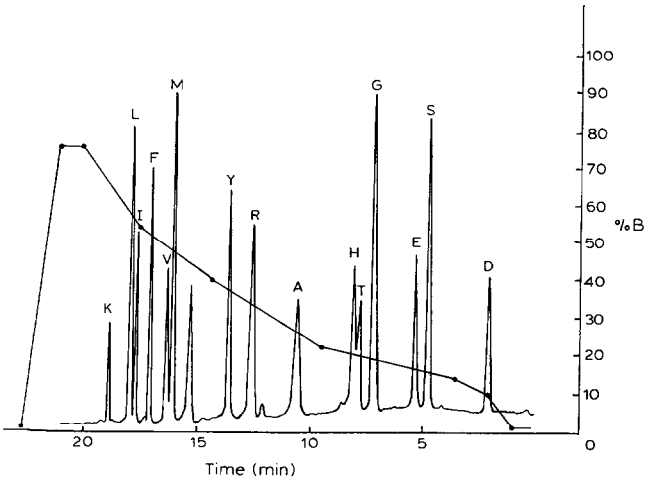

Fig. 1. Chromatogram of an amino acid standard mixture (Pierce standard H) on the Perkin-Elmer 3x3 ODS column. Buffer A, 1% THF and 2% acetonitrile in 25 m*M* sodium acetate (pH 5.4); buffer B, methanol; flow-rate, 1 ml/min. The gradient used is plotted on the chromatogram. The peak following tyrosine is believed to be an oxidation product of OPA. It increases in size as the OPA solution gets older. The one-letter code for amino acids is used.

0.1 *M* sodium acetate (pH 7.0)[18], to lower the pH of the OPA reaction mixture prior to injection. In the author's experience, addition of 50 μl of 0.13 *M* sodium phosphate (pH 3.5) to 50 μl of the OPA reaction mixture lowered the pH to only 8.5, but a volume of 1.3 *M* sodium phosphate (pH 3.5) equal to the volume of OPA reagent in 1 *M* potassium borate (pH 10.4) lowered the pH to *ca.* 6.5. When 0.5 *M* potassium borate was used as the OPA diluent, half the volume of sodium phosphate was sufficient to lower the pH to *ca.* 7. Sodium phosphate had been omitted in the earlier attempts to resolve the OPA–amino acid adducts on both columns. After *ca.* 50 injections into the PE 3x3 column, the packing material of the column dissolved. This was not observed with the Axxichrom column. The addition of sodium phosphate to reaction mixtures prior to injection appears to have stabilized and protected the columns from damage. Roth[1] has reported that phosphate quenches the fluorescence of the OPA–β-ME–amino acid adducts. The author has observed *ca.* 50% quenching under the present condition. However, even with the addition of the sodium phosphate, the sensitivity was sufficient to detect 1 pmol of each amino acid in the Standard H. Hence, sodium phosphate was added to the OPA reaction mixture to preserve the columns. The Axxichrom column has been used for over 300 chromatographic runs and still maintains good resolution. Moreover, the effect of the sodium phosphate on the retention times of the amino acids was not significant.

Purity of OPA

Both the fluoraldehyde reagent solution (Pierce) and the OPA solution, prepared as described under Materials and methods, were used. It was found that the OPA solution, prepared freshly every week, gave greater sensitivity and much lower backgrounds. In addition, some freshly opened batches of the fluoraldehyde reagent solution showed off-scale peaks in the blanks. This was overcome by the daily addition of optimum amounts of β-ME to the fluoraldehyde solutions. Typically, 20 μl of β-ME added to 400 μl of fluoraldehyde reagent solution was sufficient to minimize these fluorescent oxidation products of the OPA. However, there were several peaks in the blanks for these preparations relative to the OPA solution, prepared freshly every week. Hence, the OPA solutions, prepared freshly every week, were used for subsequent chromatographic experiments. The addition of 50 μl of OPA–β-ME reagent to the standard was sufficient to produce linear increases in fluorescence from 10 to 100 pmol of each amino acid in the mixture (Fig. 2).

Analysis of somatostatin

The results of derivatizing and injecting 6 pmol of hydrolyzed porcine somatostatin are presented in Table II and Fig. 3. As noted in Materials and methods (*Acid hydrolysis of peptides*), 20 pmol of somatostatin were hydrolyzed and the hydrolyzed peptide was dissolved in 40 μl of water. Then 12 μl (6 pmol) of this peptide were derivatized and injected into the HPLC system. From the results it is clear that quantitation at the 6 pmol level is excellent. The amount injected is recovered quantitatively in the amino acids Glu, Gly, Tyr and Leu, the remaining amino acids being higher multiples of these. The agreement of the relative numbers of amino acids with the expected ratios is excellent, with the exception of lysine, as discussed below. Also, a peptide that is oxidized with performic acid gives good quantitation for half-cystine detected as cysteic acid.

At present, the author has no explanation for the high proportion of lysine in

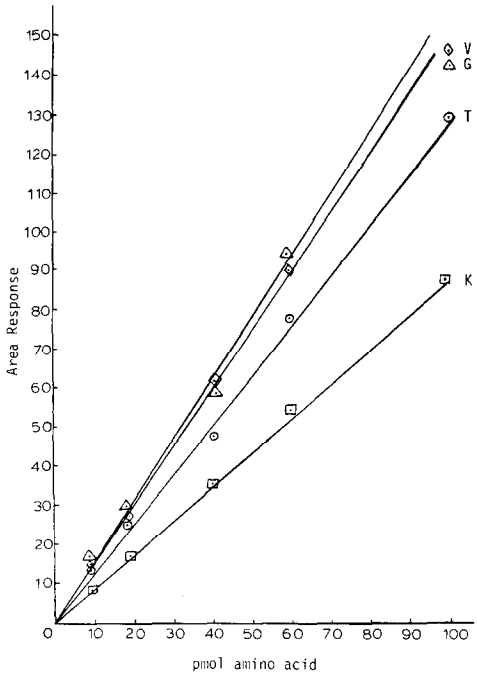

Fig. 2. Plot of the fluorescence response of some amino acids as a function of amount. The one-letter code for amino acids is used.

TABLE II

RESULTS OF DERIVATIZING AND INJECTING SOMATOSTATIN, FOLLOWING HYDROLYSIS

Amount hydrolysis, 20 pmol; amount injected, 6 pmol. n.d. = not detected.

Amino acid	*Amount obtained (pmol)*	*Relative molar ratio**	*Expected molar ratio*
Asp (+ Asn)	15.3	2.9	3
Glu (+ Gln)	6.1	1.2	1
Ser	15.1	2.9	3
Gly	6.7	1.3	1
Thr	11.2	2.2	2
Arg	11.8	2.3	2
Ala	20.8	4.0	4
Tyr	6.3	1.2	1
Phe	10.5	2.0	2
Leu	5.2	1.0	1
Lys	15.8	3.0	1
Cys** (as CA)	12.5	2.4	2
Pro	n.d.	—	2

* The relative molar ratio was obtained by multiplying the values in column 2 by a factor which would yield values for column 3 as close to integers as possible.

** The value for Cys was obtained by correlating it with the value of Phe obtained for peptide that was oxidized with performic acid and then hydrolyzed.

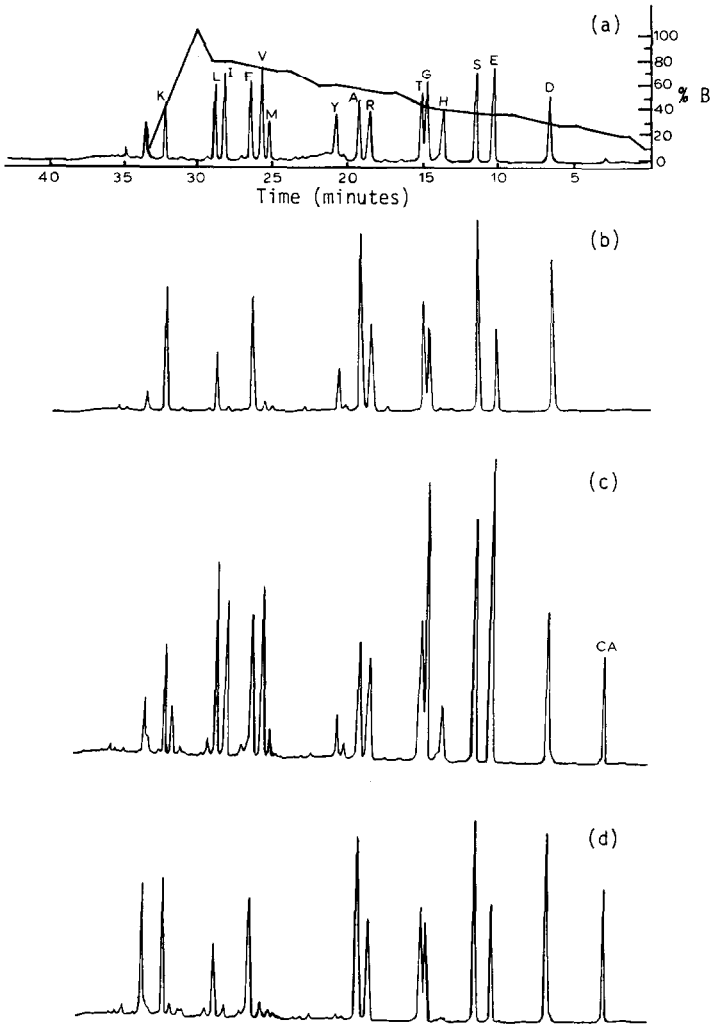

Fig. 3. Chromatograms of hydrolyzed standards and somatostatin samples injected on the Axxichrom 5 μm column. Buffer A, 1% THF in 25 m*M* sodium acetate (pH 5.6); buffer B, methanol; flow-rate, 1.0 ml/min. The gradient used is shown for chromatogram (a), which was obtained from 7 pmol of each amino acid (Pierce standard H) derivatized and injected. The other chromatograms are: (b) 6 pmol somatostatin, derivatized and injected; (c) 7.5 pmol amino acid standard, derivatized and injected following performic acid oxidation and hydrolysis; (d) 6 pmol somatostatin, derivatized and injected following performic acid oxidation and hydrolysis.

the peptide. It should be pointed out that the same results were obtained when 1 nmol and 200 pmol of the peptide were hydrolyzed. Also, correct proportions of lysine were obtained on hydrolyzing other lysine-containing peptides (data not shown). The response for the OPA derivative of lysine is quite linear, as can be seen from Fig. 2.

For the quantitation of cysteine (as cysteic acid), its value was calculated relative to that of Phe. The value obtained for amino acids such as Phe and Leu were

virtually unchanged when compared with the sample that had not been oxidized with performic acid.

The derivatization of proline with OPA following treatment with chloramine T and sodium borohydride[19] shows promise as a routine analytical procedure. It is currently being investigated by the author.

In the analysis of picomole amounts of peptides, the value of hydrolyzing the peptide in a properly evacuated chamber, devoid of an oxidizing atmosphere, must be stressed. It has been the author's experience that more errors are introduced in the quantitation by improper hydrolysis conditions than by any other single variable.

This method has also been used to analyze other peptides that have been purified from HPLC using reversed-phase solvents such as acetonitrile and aqueous trifluoracetic acid. Typically, 20–30 pmol were used for acid hydrolyses and reliable quantitation obtained at the 5–10 pmol level (data not shown).

It should be pointed out that although the OPA method has been proposed as having great sensitivity (in the femtomole range) it has not been investigated in this range. Several researchers in the field[13,14,16,18] have proposed great sensitivity for the method, but inject only a portion of the derivatized sample into the HPLC system. This has the effect of attenuating the contaminant peaks that could arise from the derivatizing reagent. The author, on the other hand, injects the entire derivatized samples into the HPLC system. In addition, although hydrolysis is carried out with picomole amounts of peptides by these investigators, the reports give very few details about the methods they used. The problem arises when only a small amount of peptide is initially available and has to be hydrolyzed and quantitated in a reliable manner. This is the problem that is addressed in this paper.

The work presented in this paper is by no means the lower limit of this method, but certainly allows any investigator several opportunities to obtain the amino acid composition of a peptide by injecting 6 pmol at a time from a hydrolyzed sample containing 20 pmol.

In summary, the OPA method optimized as described in this paper provides excellent results for amino acid composition analyses of picomole amounts of proteins and peptides. This is of value especially to the recombinant DNA industry and in biochemical research where characterization of very low amounts of peptides and proteins is often necessary.

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