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Glycerol affects the quantification of aspartate and glutamate in acid-hydrolyzed proteins

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Summary. Glycerol is widely used in protein isolation pathways to improve folding and solubility of the proteins of interest. Amino acid composition analysis of protein samples hydrolyzed in the presence of glycerol resulted in underestimation of aspartate and glutamate, when compared to hydrolysis in the absence of glycerol. Quantification of free asparagine, aspartic acid, glutamine and glutamic acid hydrolyzed with hydrochloric acid or methanesulfonic acid in the presence of glycerol resulted in poor recoveries of aspartate and glutamate (between 6 and 66%). Gas chromatography-mass spectrometry analysis of the hydrolyzates revealed, as expected, the presence of esterification products. The esters were formed between the primary and secondary hydroxyl groups of the glycerol and both carboxyl groups of the amino acids. Protein samples intended for compositional analysis should be free of glycerol.

Keywords: Amino acid analysis – Hydrolysis – Glycerol – Aspartate – Glutamate – Protein quantification

Abbreviations: EI, electron impact; GC-MS, gas chromatography-mass spectrometry; MS, mass spectrum; MSA, methanesulfonic acid; BSTFA, bis-(trimethylsilyl)trifluoroacetamide; TMS, trimethylsilyl

Introduction

Glycerol is an important reagent in Organic Chemistry and the starting material for many industrial products. In Biochemistry, glycerol is widely used in protein isolation pathways, usually at concentrations 5–25%, to facilitate folding and to increase protein solubility and stability (Sousa, 1997). Quality control studies of purified proteins include amino acid composition analysis, a classical, rather complex analytical technique, which is indispensable for protein quantification. Amino acid analysis is also used for identification

by database search of proteins separated by one- and two-dimensional polyacrylamide gel electrophoresis (Wilkins et al., 1996; Fountoulakis et al., 1997).

Prior to compositional analysis, proteins have to be hydrolyzed to liberate their residues. Hydrolysis is usually performed with hydrochloric acid or methanesulfonic acid and is a critical step of the analytical process (Spackman et al., 1958; Blackburn, 1978; Weiss et al., 1997). Presence of various stabilizing or bacteriostatic agents in the protein sample buffer, such as sodium chloride or sodium azide, can affect the quantification of certain residues, such as tyrosine, methionine, and cysteine (Manneberg et al., 1995a), and consequently falsify the identification and quantification of the proteins of interest. We reported the use of low concentrations of sodium azide during acid hydrolysis for the quantification of cysteine residues (Manneberg et al., 1995b).

In recent experiments we observed that amino acid composition analysis of recombinant proteins hydrolyzed with HCl in the presence of glycerol yielded reduced ratios for aspartic acid and glutamic acid compared to the expected values. These residues were correctly identified when hydrolysis was performed in a buffer free of glycerol. Here, using free amino acids, we show that presence of glycerol in the hydrolysis mixture leads to underestimation of aspartate and glutamate.

Materials and methods

Materials

Asparagine, aspartic acid, glutamine and glutamic acid were obtained from Sigma. Glycerol p.a. was from Fluka. Hydrolysis agents, hydrochloric acid and methanesulfonic acid, were purchased from Pierce. Recombinant transcription factor σ^{42} from *Streptococcus pneumoniae* was expressed and purified from *E. coli*.

Amino acid composition analysis

The amino acid analysis was performed following liquid-phase hydrolysis essentially as described (Fountoulakis et al., 1997; Weiss et al., 1997). In short, 25 μl of the protein in 50 mM Tris-HCl, pH 7.7, 100 mM NaCl, 1 mM $MgCl_2$, and 10 mM $ZnCl_2$ or free amino acids in 50 mM Tris-HCl, pH 7.7, 100 mM NaCl, containing or not 10% glycerol, were mixed with 1 ml of 6 M HCl or 4 M methanesulfonic acid (MSA). To the HCl solution, 0.02% phenol was added (w/v, added as solid) and in the MSA solution, 0.2% 3-(2-aminoethyl)indole was included. The solution was frozen in dry ice and evaporated below 1.3 Pa for 3 min. The hydrolysis vial was sealed under vacuum and placed in an electrical oven at 110°C for 24 h. After that time, the liquid was evaporated over NaOH and the dried hydrolyzate was dissolved in 60 μl of sodium diluent 2.20 buffer (Pickering Laboratories). Further dilutions with the same buffer were performed where necessary. Fifty μl were automatically injected into a Dionex BioLC amino acid analyzer and the residues after separation on a sodium cation exchange column (Pickering Laboratories), developed with a gradient of sodium eluents 3.15 and 7.40 (Pickering Laboratories), were derivatized with o-phthalaldehyde at 0.36 ml/min. Amino acid standards 200, 100 and 25 pmol (Hewlett Packard) were run with the samples. The peak areas were integrated using the instrument supplier's software.

Gas chromatography-mass spectrometry

Asn, Asp, Gln and Glu were hydrolyzed with 6M HCl or 4M MSA as described above. The dried hydrolyzates were dissolved in 200μl of pyridine containing 100μl of bis(trimethylsilyl)trifuoroacetamide (BSTFA) at room temperature. The reaction products were injected into a gas chromatograph (Hewlett-Packard, model 5890A) at 270°C. The stationary phase was methylsilicon (DB-1 fused-silica column, 0.25 × 15,000mm). The carrier was helium at a flow rate of 0.5m/s. A heating program was applied to the column (70–340°C, heating rate 4°C/min). Ionization was performed by electron impact (EI) at 70eV and 250°C. The analysis was performed with a Hewlett-Packard mass spectrometer (model 5989B) directly connected with the gas chromatograph.

Results and discussion

Amino acid composition analysis is one of the most reliable methods for protein quantification (Fountoulakis et al., 1992). The method comprises two steps, hydrolysis of the substrate and chromatographic analysis of the hydrolyzate (Spackman et al., 1958; Weiss et al., 1997). Proper performance of both steps is a prerequisite of a confident analysis. The presence of various stabilizing factors in the sample solution, which are useful during protein isolation, may influence the analysis. Under the conditions of the hydrolysis, these substances may react with certain amino acids and consequently affect the quantification of these residues.

Amino acid analysis of recombinant transcription factor σ^{42} hydrolyzed in a buffer containing 10% glycerol resulted in determination of lower ratios for aspartate and glutamate, 63% and 10% of the expected values, respectively, whereas the other residues were correctly determined (Table 1). N-terminal sequence analysis and mass spectrometric analysis showed that the protein had the expected N-terminal residues (A-T-K-Q-K-E-V-) and the expected molecular mass (observed mass 41,900.3Da, theoretical mass 41,898.8Da).

Table 1. Quantification of aspartate and glutamate residues of transcription factor σ^{42} in the absence and presence of glycerol[a]

Residues	Residues in protein		
	Theoretical	Observed	
		Glycerol	
		−	+
Ala	25.0	25.0	25.0
Asx	45.0	47.7	28.4
Glx	58.0	58.4	6.0

[a]Transcription factor σ^{42} was hydrolyzed with 6M HCl at 110°C for 24h in 50mM Tris-HCl, pH 7.7, 100mM NaCl, 1mM $MgCl_2$ and 10mM $ZnCl_2$, containing or not 10% glycerol. The values were normalized to the Ala residues. The ratios determined for the other residues were very close to the expected values (not shown).

On the other hand, the results of the compositional analysis were reproducible, so that we searched for the factor which caused the inconsistency in the quantification of the mentioned residues. When the protein was hydrolyzed in the same buffer but free of glycerol, analysis of the hydrolyzate yielded the expected ratios for Asx and Glx (Table 1).

Figure 1 shows partial chromatographic analyses of the hydrolysis products of transcription factor σ^{42} hydrolyzed in a buffer containing or not 10% glycerol. When the protein was treated in the absence of glycerol, only peaks corresponding to the known amino acids standards (Fig. 1A) were detected (Fig. 1B). Hydrolysis in the glycerol-containing buffer yielded additional peaks which did not correspond to standard amino acids (Fig. 1C, the peaks with retention times of 8.41 and 10.36 min). The elution profiles of the remaining residues of the protein were the same in both cases independent of the presence of glycerol (not shown). The protein composition determined fol-

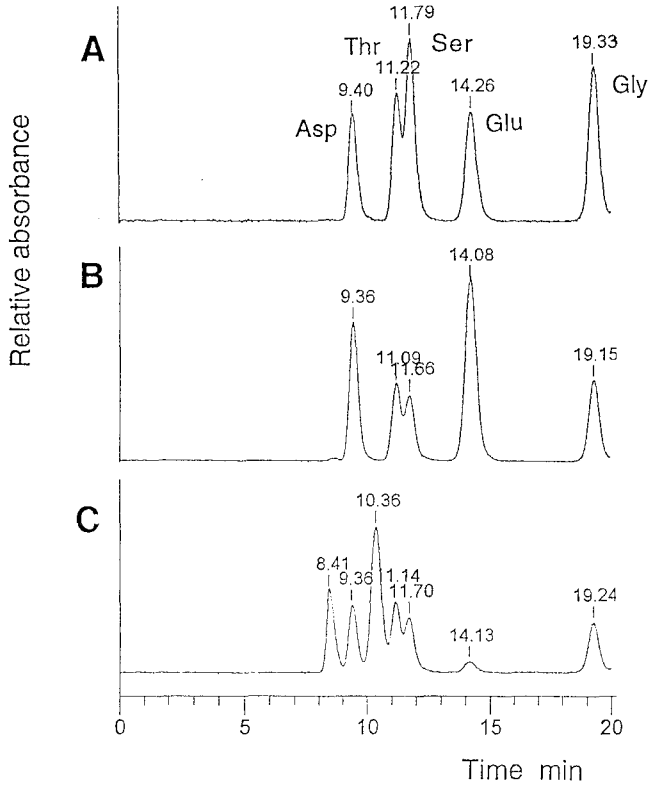

Fig. 1. Partial elution profiles of (**A**) amino acid standards, (**B**) the hydrolysis products of transcription factor σ^{42} treated in the absence of glycerol, and (**C**) the hydrolysis products of transcription factor σ^{42} hydrolyzed in the presence of glycerol. Recombinant transcription factor σ^{42} was hydrolyzed with 6 M HCl in a buffer containing or not 10% glycerol. Chromatography of the residues was performed as stated under Materials and methods. The elution profiles of the first five residues, Asp, Thr, Ser, Glu and Gly, are shown. Following hydrolysis in the absence of glycerol, only peaks corresponding to standard amino acids were detected. In the presence of glycerol, two additional signals were observed

lowing hydrolysis in the presence of glycerol was in relatively good agreement with the theoretical one (with the exception of Asx and Glx), whereas the protein amount was approximately 75% of that determined in the absence of glycerol.

In order to confirm that the products represented by the new peaks, detected in the hydrolyzate of the protein treated in the presence of glycerol (Fig. 1C), were derived from the Asx and Glx residues, we hydrolyzed free asparagine, aspartic acid, glutamine and glutamic acid in the absence and in the presence of glycerol. Fig. 2 shows the elution profiles of the substrates and of their hydrolysis products. Asparagine (Fig. 2A) was detected as aspartic acid when hydrolyzed in the absence of glycerol (Fig. 2B). However, after hydrolysis in the presence of 10% glycerol, one additional peak with a retention time of 8.49 min, corresponding to a new product was detected next to aspartic acid, which was represented by a smaller peak (Fig. 2C). Aspartic acid (Fig. 2D) migrated at the same position like the starting material when hydrolyzed in the absence of glycerol (Fig. 2E). Hydrolysis in the presence of glycerol resulted in the detection of two peaks, a smaller peak corresponding to aspartic acid and a larger one (Fig. 2F) of a similar retention time with the new product of Asn (Fig. 2C).

Similarly, additional peaks were detected in the hydrolyzates of glutamine (Fig. 2I) and glutamic acid (Fig. 2L) in the presence of glycerol. Whereas in the case of aspartate one unknown product was detected (Fig. 2C and 2F), in the case of glutamate, two unknown products with shorter retention times than Glu were observed (Fig. 2I and 2L). The peak corresponding to Glu (retention time approximately 14.13 min) was significantly smaller in comparison with the starting material (Fig. 2G and 2J) and with one of the new products with a retention time of about 10.40 min; Fig. 2I and 2L). Quantification of aspartate and glutamate in the hydrolysis products generated in the presence of glycerol, showed that 26–44% of the aspartate and only 6–31% of the glutamate were detected as such (Table 2).

The retention times of the peaks detected in the hydrolyzates of aspartate when hydrolyzed in the glycerol-containing buffer (about 8.45 min; Fig. 2C and 2F), approximately corresponded to the retention time of the first additional peak detected in the hydrolyzate of the transcription factor σ^{42} following hydrolysis in the presence of glycerol (8.41 min; Fig. 1C). Similarly, the retention time of the large peak derived from glutamate (about 10.40 min, Fig. 2I and 2L) corresponded to the peak with a retention time of 10.36 min of the hydrolyzate of the protein (Fig. 1C). The small peaks seen in Fig. 2I and 2L with an approximate retention time of 8.70 min probably commigrated with larger peaks and are not visible in Fig. 1C. Asn, Asp and Glu were also hydrolyzed with methanesulfonic acid in the presence of glycerol. The aspartate and glutamate recoveries were lower than those obtained in the absence of glycerol but significantly higher compared to hydrolysis with HCl (Table 2).

The new products detected in the hydrolyzates of Asn, Asp, Gln and Glu in the presence of glycerol were analyzed by gas chromatography-mass spectrometry (GC-MS). We have used this technique in the past

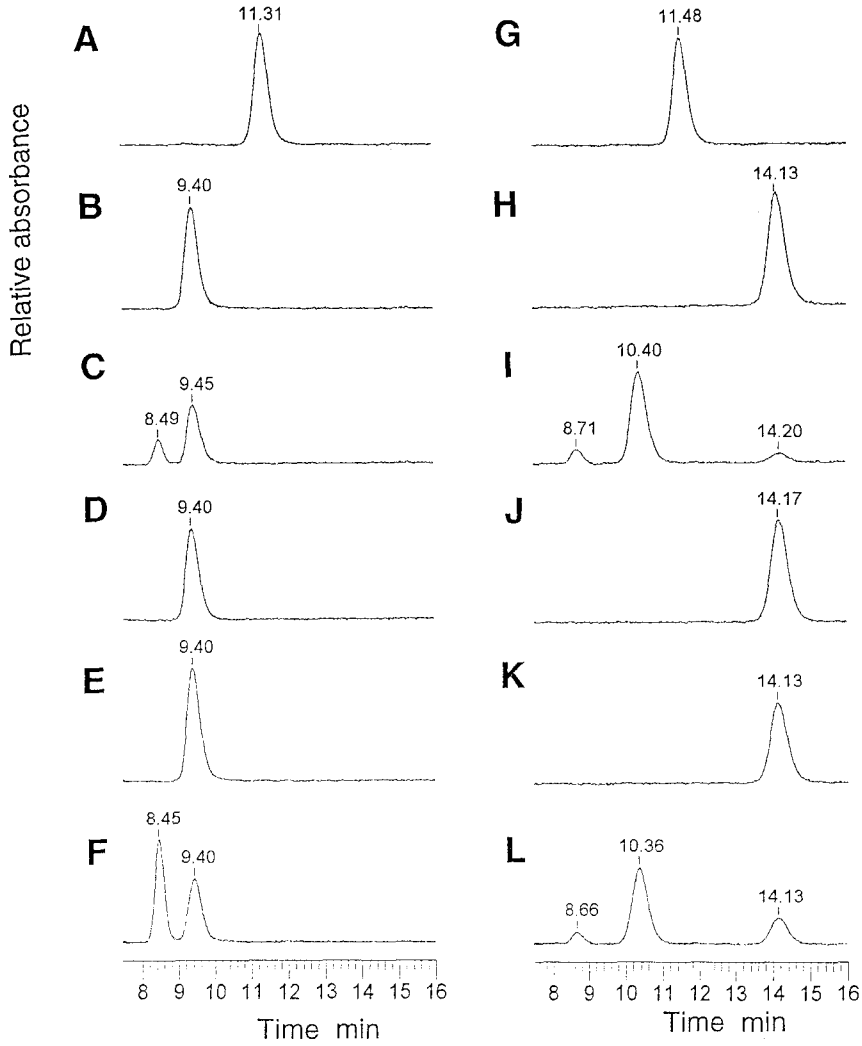

Fig. 2. Elution profiles of Asn, Asp, Gln and Glu and of their hydrolysis products in the absence and presence of glycerol. The same amounts of the mentioned residues were hydrolyzed with 6 M HCl in a buffer containing or not 10% glycerol. Chromatography of the residues was performed as stated under Materials and Methods. **A**, **C**, **G**, and **J**, elution profiles of untreated Asn, Asp, Gln and Glu, respectively. **B**, **E**, **H**, and **K**, elution profiles of Asn, Asp, Gln and Glu, respectively, hydrolyzed in the absence of glycerol. **C**, **F**, **I**, and **L**, elution profiles of Asn, Asp, Gln and Glu, respectively, hydrolyzed in the presence of glycerol. In the presence of glycerol, additional signals were detected in the hydrolyzates

to identify protein adducts (Fountoulakis et al., 1995). Figure 3 shows a GC-MS analysis of the hydrolysis products of Asp after reaction with bis-(trimethylsilyl)trifluoroacetamide. For the products represented by peaks *b* and *c* (Fig. 3A) masses of 495 Da were found (Fig. 3B and 3C, respectively), corresponding to the trimethylsilyl (TMS) derivatives of esters formed between one molecule of aspartic acid and one molecule of glycerol ($115 + 92 + 4 \times 72 = 495$).

Table 2. Quantification of Asn, Asp, Gln and Glu following hydrolysis in the presence of glycerol[a]

Residue	Hydrochloric acid	Methanesulfonic acid
Asn	27	46
Asp	45	56
Gln	7	ND
Glu	31	66

[a] Asparagine, aspartic acid, glutamine and glutamic acid in a buffer, containing or not 10% glycerol were hydrolyzed with 6M HCl or 4M methanesulfonic acid at 110°C for 24h. Following hydrolysis, Asn and Gln were recovered as Asp and Glu, respectively. Quantification of the residues was performed in comparison with amino acid standards in a second run and is expressed as percentage of the values recovered after hydrolysis in the absence of glycerol (100%).

The mass of 232Da detected in the electron impact-mass spectrum (EI-MS) (Fig. 3B) of peak *b* (Fig. 3A) corresponds to the TMS-derivative of the fragment of the aspartic acid-glycerol ester within the frame (Fig. 4B), suggesting that the carboxyl group on the Cα atom of aspartic acid participated in the ester formation with glycerol. In the EI-MS of the substance represented by peak *c* (Fig. 3A), the mass of 218Da (Fig. 3C) corresponds to the TMS-derivative of the fragment shown in the window of Fig. 4C. In the compound shown in Fig. 4C, the carboxyl group of the side chain of the aspartic acid is involved in the ester formation. The difference between the masses of the two fragments in the windows of Fig. 4B and 4C is 14Da, which corresponds to the mass of the additional methylene group of the fragment of Fig. 4B. Thus, the esterification took place at both carboxyl groups of the aspartic acid. In addition to the monoesters, diglycerol esters were detected in the hydrolyzate as well, like the compound represented by peak *d* (Fig. 3A). Similar products were detected in the hydrolyzate of Asn. Larger amounts of the glycerol ester were detected in which the carboxyl group of the side chain of the Asp (resulted from the hydrolysis of Asn) was involved (data not shown). The various esters produced during hydrolysis were probably not resolved by the ion exchange chromatography and migrated as one peak with a retention time of about 8.45min (Fig. 2C and 2F).

In the hydrolyzates of Gln and Glu, mono- and di-glycerol esters with glutamic acid were also detected. In addition, a peak with a mass of 273Da was detected, corresponding to the TMS-derivative of 5-oxoproline, a cyclization product of glutamic acid carrying a lactam ring (data not shown). Moreover, in the EI-MS of the TMS-derivatives of the hydrolysis products of Gln and Glu, peaks were detected corresponding to glycerol esters with 5-oxoproline and glutamic acid, in which the hydroxyl group on the C2 atom of glycerol was involved. Esters involving the secondary hydroxyl group of glycerol were formed with both carboxyl groups of Glu (data not shown). The amount of these esters was smaller in comparison with those involving the

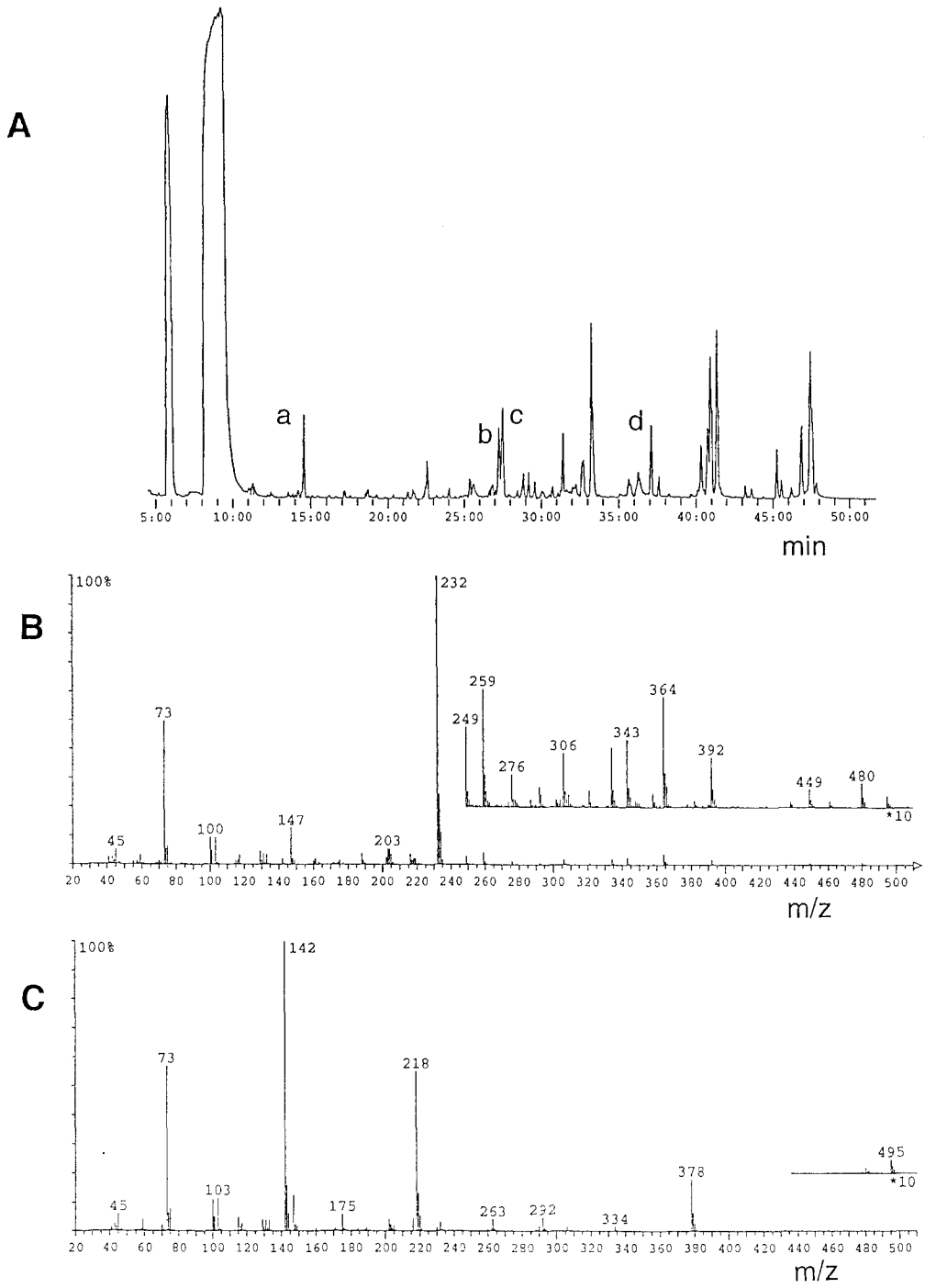

Fig. 3. Gas chromatography-mass spectrometry analysis of the TMS-derivatives of the hydrolysis products of aspartic acid. Aspartic acid was hydrolyzed with 6M HCl in the presence of 10% glycerol for 24h. The dried hydrolyzate was dissolved in pyridine and treated with BSTFA. Analysis was performed as stated under Materials and methods. **A** Reconstructed total ion current of the hydrolyzate derivatives. The two first large peaks correspond to glycerol. Peak *a* corresponds to Asp; peaks *b* and *c* represent monoglycerol esters with Asp (the compounds shown in Fig. 4B and 4C, respectively), and peak *d* represents a diglycerol ester with Asp. **B**, **C** Electron impact mass spectra of TMS-derivatives of the esterification products represented by peaks *b* and *c* of Fig. 3A, respectively

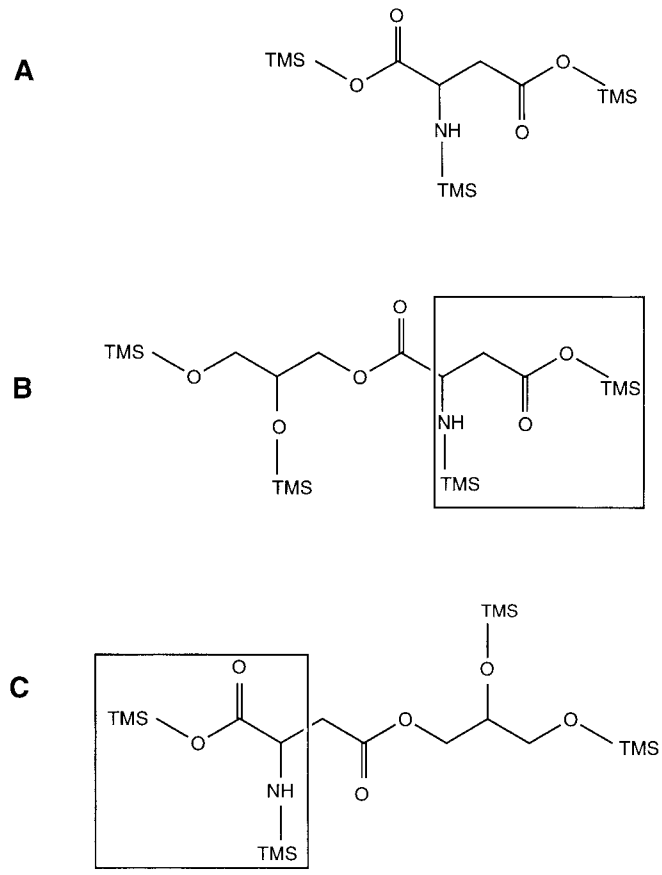

Fig. 4. Trimethylsilyl derivatives of hydrolysis products of aspartic acid. **A** TMS-derivative of Asp (represented by peak *a*, Fig. 3A). **B** TMS-derivative of the ester between the primary hydroxyl group of glycerol and the carboxyl group at the Cα atom of Asp (represented by peak *b*, Fig. 3A). **C** TMS-derivative of the ester between the primary hydroxyl group of glycerol and the carboxyl group of the side chain of Asp (represented by peak *c*, Fig. 3A). **B C** The structures in the windows are fragments of the esters with masses 232 and 218 Da (mass spectra in Fig. 3B and 3C, respectively)

primary hydroxyl groups. The two peaks with retention times of about 8.70 and 10.40 min, seen in the chromatograms of the hydrolyzates of Gln and Glu (Fig. 2I and 2L, respectively), probably correspond to esters of Glu with the hydroxyl groups on the C2 and C1 atoms of glycerol, respectively. Esters involving the secondary hydroxyl group of glycerol were not clearly detected in the hydrolyzates of Asn or Asp.

Here only the hydrolysis products of Asn, Asp, Gln and Glu generated in the presence of glycerol were analyzed. It is possible that small amounts of glycerol esters with the Cα carboxyl groups of the other amino acids were formed as well. This could explain the underestimation of the protein amount in the presence of glycerol. However, no additional peaks corresponding to new products were detected in the chromatograms (except of the mentioned ones).

In summary, we provide evidence that hydrolysis of proteins with HCl or methanesulfonic acid in buffers containing glycerol results in underestimation of aspartate and glutamate. Using free amino acids, we found that during acid-hydrolysis a wide spectrum of esterification products, involving many reaction modes between both carboxyl groups of the amino acids and primary and secondary hydroxyl groups of the glycerol, were generated. The generation of the amino acid side products with glycerol is responsible for the reduced ratios of aspartate and glutamate detected by compositional analysis. Protein samples intended for amino acid composition analysis should therefore be free of glycerol.

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