Sequential GC/MS Analysis of Sialic Acids, Monosaccharides, and Amino Acids of Glycoproteins on a Single Sample as Heptafluorobutyrate Derivatives

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ABSTRACT: A GC/MS procedure was developed for the analysis of all major constituents of glycoproteins. The rationale for this approach is that by using GC/MS analysis of the constituents as heptafluorobutyrate derivatives, it was possible to quantitatively determine the sialic acid, monosaccharide, fatty acids (when present), and the amino acid composition with the sample remaining in the same reaction vessel during the entire procedure. A mild acid hydrolysis was used to liberate sialic acids and was followed by formation of methyl-esters of heptafluorobutyrate (HFB) derivatives. After GC/MS analysis of sialic acids, the remaining material was submitted to acid-catalyzed methanolysis followed by the formation of HFB derivatives. After GC/MS analysis of the monosaccharides, the sample was supplemented with norleucine (as internal standard) and hydrolyzed with 6 M HCl followed by the formation of isoamyl-esters of HFB derivatives and GC/MS analysis. His and Trp residues were modified during the step of acid-catalyzed methanolysis, but the resulting derivatives were stable during acid hydrolysis and quantitatively recovered by GC/MS analysis. As a result, all constituents of glycoproteins (sialic acids, monosaccharides (or di- and trisaccharides) and amino acids) are identified in the electron impact mode of ionization and quantified using three GC/MS analysis in the same chromatographic conditions and using a limited number of reagents, a considerable advantage over previous techniques. This method is very sensitive, all data (qualitative and quantitative) being obtained at the sub-nanomolar level of initial material.

In previous studies (*1*−*3*), we have demonstrated that the complete analysis of the constituents of glycolipids could be performed by GC/MS analysis of heptafluorobutyrate derivatives of the various constituents liberated using acid-catalyzed methanolysis. During this procedure, the released monosaccharides are transformed into their *O*-methyl glycosides and *O*-methyl glycosides of their methyl esters, fatty acids as their methyl esters (FAMEs)[1] and long-chain bases as free compounds or as degradation products that could be unambiguously identified. High-temperature acylation with heptafluorobutyric anhydride allows complete blockage of alcohol and amino groups as heptafluorobutyrate derivatives, after which all constituents become volatile and suitable for GC analysis. The assignment of relative molar response factors for the various compounds using the total ion counts in the electron impact mode (*3*) allowed the molar compositions of glycosphingolipids to be obtained in a reproducible way with the sample remaining in the same test tube during all cleavage/derivatization steps. The same methodology was successfully applied to study the diversity of sialic acids (*4*), and 38 different sialic acid derivatives could be identified using GC/MS analysis. For gangliosides, it was possible to first analyze the sialic acids, and after methanolysis of the remaining material to obtain a complete quantitative determination of all monosaccharides, fatty acid methyl-esters, long-chains bases, again with the sample remaining in the same tube.

Here, we have adapted these approaches to analyze glycoproteins—a challenging task considering the potential complexity of these macromolecules. Indeed, previous approaches needed several techniques and several apparatus, such as HPLC and/or gas chromatography for the separation of the different constituents in separate experiments. Due to difficulties of coupling such techniques with mass spectrometry, the different compounds were mainly identified through their retention time, a criterion largely insufficient for analyzing samples of unknown composition. In contrast, GC/MS analyses of stable volatile compounds offered the possibility to analyze complex mixtures (*1*−*4*) because the nature of the constituent of each peak can be certified by its

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[1] Abbreviations: *amu*: atomic mass unit; CI: chemical ionization; EI: electron impact ionization; FAMEs: fatty acid methyl-esters; GC: gas chromatography; MS: mass-spectrometry; HFB: heptafluorobutyrate; HFBAA; heptafluorobutyric anhydride; Kdn: 3-deoxy-D-glycero-D-galacto-nonulosonic acid; RT: retention time; rMR: relative molar response; rRT: relative retention time; TIC: total ion count; Uro: uromodulin. The nomenclature of the other sialic acids was after Schauer and Kamerling (*12*).

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mass spectrum. In this paper, we report that complete analysis of glycoproteins (sialic acids, monosaccharides, and amino acids) is achieved using three GC/MS analysis steps in the same chromatographic conditions and the same mode of detection (electron impact ionization) starting from subnanomolar quantities of material. New information on the electron impact MS fragmentation patterns of the heptafluorobutyrate derivatives of the isoamyl-esters of amino acids are provided. Especially, Trp, which is modified during the methanolysis step, can be quantitatively recovered after acid hydrolysis. Results obtained from glycoprotein mixtures as well as from purified glycoproteins are reported.

EXPERIMENTAL SECTION

Chemicals and Biological Materials. Diazogen was from Acros and heptafluorobutyric anhydride (HFBAA, puriss. grade) from Fluka or from Merck or from Acros. Methanol and isoamyl alcohol were redistilled in anhydrous conditions from analytical grade reagents. Standard amino acids were from Beckman and Pierce. MUC2 was isolated as an insoluble glycoprotein complex from human colon and purified as reduced subunits with isopycnic density-gradient centrifugation as described (*5*). Uromodulin (Uro) was isolated according to Serafini-Cessi et al. (*6*).

GC/MS Analysis of Sialic Acids. Glycoprotein-bound sialic acids were analyzed as previously described (*4*). Briefly, samples (1−10 μg of protein) were hydrolyzed (105 min at 80 °C in 500 μL of 2 M acetic acid), and then lyophilized or evaporated with a rotary evaporator at room temperature. The dry samples were supplemented with 200 μL of anhydrous methanol followed by the addition of a diazomethane solution in diethyl-ether (200 μL) and left overnight at room temperature after vigorous agitation. It is emphasized that diazomethane is a very potent carcinogenic and that all handling procedures must be performed accordingly (*4*). Before GC/MS analysis, samples were evaporated to dryness under a stream of nitrogen, supplemented with 200 μL of acetonitrile and 25 μL of heptafluorobutyric anhydride (HFBAA) and heated for 5 min at 150 °C in a sand bath. After cooling, samples were evaporated, dissolved in 400 μL of acetonitrile dried on calcinated calcium chloride and an aliquot (1 μL) was injected onto the Ross injector of the GC/MS. It should be emphasized that HFBAA did not derivatize the OH group of the $C_{(2)}$ carbon atom of sialic acids and was not inducing a trans-acylation of the initial acyl groups of sialic acids (*4*).

GC/MS Analysis of Monosaccharides (and of Fatty Acids). After analysis of sialic acids, samples were dried under a stream of nitrogen and submitted to acid-catalyzed methanolysis (20 h at 80 °C in 500 μL of anhydrous methanol containing 0.5 M gaseous HCl; 1, 7). The methanolysis reagent was obtained by dissolving anhydrous gaseous HCl (up to 0.5 M) at −50 °C in anhydrous methanol previously redistilled on magnesium turnings (*7*). Gaseous HCl was prepared by the dropwise addition of concentrated sulfuric acid on crystallized sodium chloride. After drying them under a stream of nitrogen, samples were supplemented with 200 μL of acetonitrile and 25 μL of heptafluorobutyric anhydride and heated for 30 min at 150 °C in a sand bath. After the reagents were evaporated under a stream of nitrogen, samples were dissolved in 200 μL of dried acetonitrile and 1 μL was injected in the Ross injector of the GC/MS. Under these conditions, all monosaccharides (including sialic acids) are recovered as *O*-methyl-glycosides except the particular glucosamine residue forming the N-glycosidic bond (*8*).

GC/MS Analysis of Amino Acids. After monosaccharide analysis, samples were dried under a stream of nitrogen and submitted to "classical" acid hydrolysis (16 h at 110 °C in 100 μL of 6 M HCl). After the addition of 1 nmol of norleucine (Nle) as internal standard, samples were evaporated under a stream of nitrogen and submitted to a short time methanolysis (500 μL of 0.5 M HCl in anhydrous methanol, 1.5 h at 80 °C) to form methyl esters (*9*). After drying them under a stream of nitrogen, samples were transesterified using 200 μL of 1.5 M HCl in redistilled isoamyl alcohol (*9*) overnight at 100 °C. The reagent was obtained by adding 107 μL of acetyl chloride in precooled (−20 °C) isoamyl alcohol. After drying them under a stream of nitrogen and mild heating with a hair drier, samples were supplemented with 200 μL of acetonitrile and 25 μL of HFBAA and heated for 30 min at 150 °C. After evaporation, samples were dissolved in 400 μL of dried acetonitrile, and an aliquot (1 μL) was injected into the Ross injector of the GC/MS. As previously discussed (*3*, *9*, *10*), methyl-esters of amino acids are too volatile to be analyzed by GC or GC/MS. In contrast, the isoamyl-esters of HFBAA derivatives will allow the excess HFBAA to be eliminated without losing the most volatile amino acids derivatives (those of Ala, Gly, Val, Thr, Ser).

GC/MS Analysis. For GC/MS analysis, the GC separation step was performed using a Carlo Erba GC 8000 gas chromatograph equipped with a 25 m × 0.32 mm CP−Sil5 CB Low bleed/MS capillary column, 0.25 μm film phase (Chrompack France, Les Ullis, France). The temperature of the Ross injector was 260 °C and samples were analyzed using a temperature program starting at 90 °C for 3 min, followed by an increase (5 °C/min) until 260 °C was reached. The column was coupled to a Finnigan Automass II mass spectrometer (mass detection limit 1000) or, for masses larger than 1000, to a Riber 10−10H mass spectrometer (mass detection limit 2000). Analyses were routinely performed in the electron impact mode (ionization energy 70 eV; source temperature 150 °C) and to preserve the filament of the ionization source, GC/MS records were performed 5 min after injection. Quantitation of the various constituents was performed using the total ion count (TIC) of the MS detector and the Xcalibur software (Finnigan Corp.). To assess the mass of the various derivatives, MS analyses were also performed in the chemical ionization mode in the presence of ammonia (ionization energy 150 eV, source temperature of 100 °C). Detection was performed for positive ions.

RESULTS AND DISCUSSION

The approach reported here has emerged through was a compilation of several lines of work on the use of heptafluorobutyrate (HFB) derivatives for the analysis of monosaccharides (*1*, *11*), constituents of complex lipids (*1*−*3*) and of sialic acids (*4*). The major advantages of HFB derivatives was their stability with time, their excellent chromatographic properties on "classical" methyl-siloxane columns (absence of van der Waals interaction with the liquid phase) and, in excess of reagent, the almost complete absence of interfer-

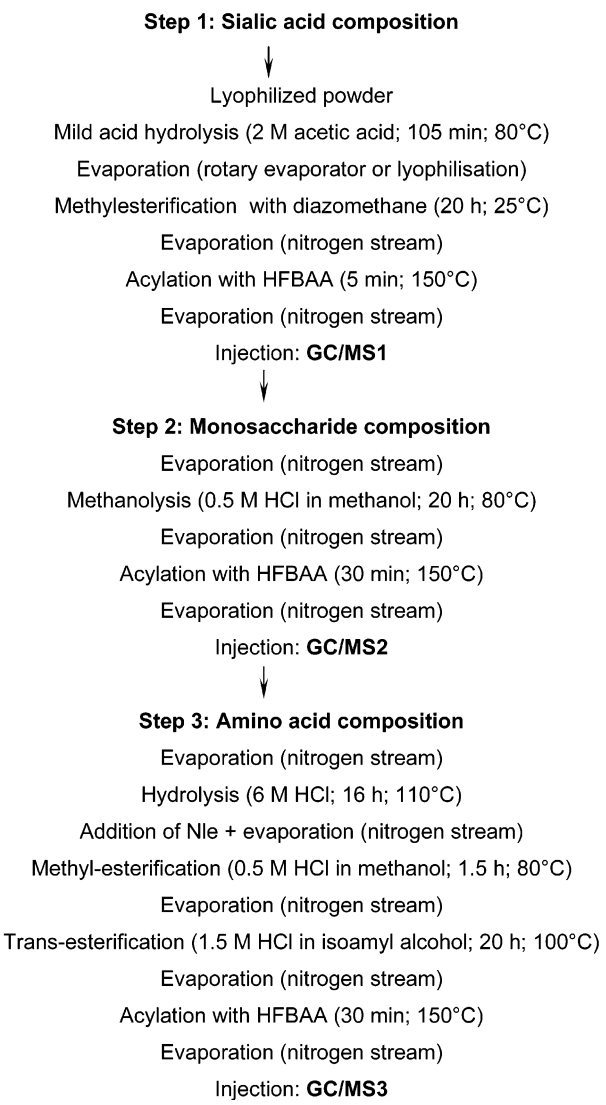

FIGURE 1: Basic principles of the approach. The approach involves three sequential GC/MS analyses (GC/MS1, GC/MS2, and GC/MS3) performed using the same apparatus, the same GC column, the same temperature program and the same mode of detection (electron impact ionization) of the same sample always remaining in the same reaction vessel, without extraction or centrifugation. The details of each protocol are reported in Materials and Methods. It is emphasized that, due to the toxicity of reagents, all handling procedures must be performed accordingly.

ence with the constituents of buffers used for the purification of glycoproteins [salts, most detergents (except SDS and octyl-glucoside), Tris and glycine (*1*)]. The HFB derivatives of these compounds (or those of the methyl-ester of Gly) are volatile to the extent that they are almost completely eliminated during evaporation of the excess of HFBAA before injection onto the GC/MS apparatus. Furthermore, HFB derivatives are quite resistant to acid-catalyzed methanolysis so that the glycosidic bond involving de-*N*-acetyl-glucosamine (found in the GlcN−Ins bond of GPI-anchored glycoproteins or glycolipids) is cleaved because the shielding effect of the initially protonated free NH_2 group is eliminated after its substitution by a HFB group.

Summary of the Approach. The basic principles of the approach are summarized in Figure 1. It involves three sequential GC/MS analyses of sialic acids, the monosaccharides, and the amino acids. As previously reported (for review, see ref *10*), the mild acid hydrolysis step liberated the major part of the sialic acids (over 95%) with no significant release of other constituents. However, GC/MS analysis indicated that monosaccharides in terminal nonreducing positions of glycans could be cleaved but to a very low extent (*4*). Possibly some particularly weak peptide bonds could also be cleaved, but since the sample remains in the same reaction vial until the end of the procedure, and components released are still available for the subsequent analyses, such cleavages will not perturb the final result. After the first hydrolysis step, the dried samples (containing free sialic acids and essentially the remaining intact glycoprotein) were methyl-esterified with an excess of diazomethane and, after elimination of the reagent, all alcohol and (possibly) amino groups were blocked by HFBAA. Sialic acids were thus recovered as volatile HFB derivatives of their methyl esters and analyzed in the step GC/MS1 (see ref *4* for the detailed mass spectra of 38 different sialic acids).

After the GC/MS1 (sialic acid analysis), the samples were submitted to acid-catalyzed methanolysis, liberating all monosaccharides from the glycoprotein as their *O*-methyl-glycosides, except the GlcNAc residue involved in N-glycosidic bonds. Indeed (*8*), the latter constituent did not behave as the other GlcNAc residues involved in *O*-glycosidic bonds (which were quantitatively recovered as *O*-methyl-glycosides), but as the 1-glycosylamine of GlcNAc. Consequently, this residue was recovered essentially as the HFB derivative of GlcN, although some minor components (HFB derivative of the glycosylamine of GlcN and HFB derivative of an Amadori rearrangement product of GlcN) were also observed (*8*). Under these conditions, the carboxyl groups of acidic monosaccharides are transformed into their methyl-esters and all O-acyl groups of sialic acids, as well as all acetamido groups of N-acylated compounds (GlcNAc, GalNAc, Neu5Ac, Neu5Gc) are cleaved forming N-deacylated compounds. After reaction with HFBAA, the monosaccharides were recovered as the per-O(N)-HFB derivatives of their *O*-methyl-glycosides (and of their methyl-esters for compounds with a carboxyl group, such as hexuronic acids and sialic acids), except the residue involved in the N-glycosidic bond. All these derivatives were quantitatively and qualitatively determined during GC/MS2. During methanolysis, the acetamido groups of Asn and Gln in the glycoprotein were transformed into Asp and Glu methyl-esters, respectively.

After the GC/MS2 step (analysis of the HFB derivatives of the *O*-methyl-glycosides), the approach allowed amino acid analysis using the same GC/MS setup. The "classical" HCl hydrolysis for proteins cleaved almost all peptide bonds (sequences of several amino acids with alkane chains were particularly resistant), but also induced some degradation of the less stable amino acids (especially Ser and Thr). Under these conditions, the HFB derivatives of the monosaccharides were in large part destroyed (except the hexosamines) and all acyl and methyl-ester groups were lost. To analyze the liberated amino acids, suitable volatile derivatives allowing reproducible quantification must be identified taking into account the stability of certain sequences and the degradation of the less stable amino acids).

As previously reported (*3*, *9*, *10*), the methyl-esters of the HFB derivatives of Ala, Gly, Val, Thr, and Ser are too volatile and largely lost when removing the excess of HFBAA). In contrast, butyl- (*9*) and isoamyl-esters (*9*)

afforded the required volatility, i.e., allowing the complete evaporation of the excess of reagent (HFBAA) and of the product (HFBA) without loss of amino acid derivatives. It should be emphasized that the same criteria were used when choosing the HFB derivatives instead of pentafluoropropionic derivatives for the GC/MS2 step (pentafluropropionate derivatives of pentoses and deoxy-hexoses were in large part lost at the step of evaporation of evaporation of the excess of reagent (*1*)). Isoamyl-esters are preferred because of their superior GC separation properties (*9*). Unfortunately, several amino acids are not soluble in 1-butanol (*10*) or in isoamyl-alcohol (*9*) impeding the quantitative formation of esters and, consequently, the recovery of volatile derivatives. In contrast, as previously reported (*9*), the appropriate derivatives could be obtained using a two-step procedure: (i) formation of methyl esters using a short-time acid-catalyzed methanolysis and (ii) a long-time acid-catalyzed trans-esterification with a longer-chain alcohol (*10*) and, for the reasons invoked above, isoamyl alcohol was chosen (*9*). Using this two-step procedure, carboxyl groups of amino acids were transformed into their isoamyl esters and after reaction with HFBAA, the O(N)-HFB derivatives of their isoamyl-esters (Figure 2). During these conditions, the monosaccharides remaining after HCl hydrolysis (essentially but not only hexosamines) were transformed into the per-HFB derivatives of their *O*-isoamyl-glycosides and could be analyzed in the third step of GC/MS analysis (GC/MS3), without interferences with the derivatives of the amino acids.

GC/MS Identifications of the HFB Derivatives. (a) Sialic Acids. GC/MS analysis of the sialic acids as HFB derivatives (including the mass spectra of 38 different sialic acids from the Neu5Ac, Neu5Gc, and Kdn families) was reported in details in a previous paper (*4*). All these compounds were shown to have different retention times (see also Figure 3a). The mass spectra associated with specific retention times (Rt) allowed the unambiguous identification of all compounds. When analyzing total homogenates from mammalian cells, the peaks of sialic acids could be safely identified among the contaminants (essentially phthalates from plastic vessels) by chromatogram reconstitution using the ion at $m/z = 169$ specific for HFB derivatives. Integration of the sialic peaks could be also performed using this ion. Because of the absence of suitable sialic acid standard, the relative molar responses (rMR) of the various sialic acid derivatives could not be determined precisely. Nevertheless, our studies of different samples (including compounds of the Neu5Ac, Neu5Gc, and Kdn series) indicated that the data obtained from the integration of the sialic acid peaks in the reconstituted chromatogram for the ion at $m/z = 169$ differed significantly from the total ion count (TIC) response only for the poly-*O*-acetylated sialic acids (5% lower estimation for di-*O*-acetylated Neu5Ac relative to Neu5Ac in the series of *N*-acetyl-neuraminic acids). In fact, for all the highly sialylated samples discussed here, the integrations were easily performed on the TIC chromatogram.

(b) O-Methyl-glycosides. The GC/MS analysis of the *O*-methyl glycosides allowed the qualitative and quantitative determination of the monosaccharide composition of glycoproteins, the different anomers of the different monosaccharide being clearly identified through their mass spectra and Rt. The details of these analyses were reported in previous papers (*1*, *2*, *8*). Common monosaccharides (Ara, Fuc, Rha, Rib, Xyl, Gal, Glc, Man, GlcA, GalA, IdoA, GlcNAc, GalNAc, Kdn, and neuraminic acid) were perfectly separated (Figure 3b), the quantitation being performed by integrating only the major anomer and attributing specific relative molar response (RMR) coefficient for the EI mode of ionization (*3*). It should be emphasized that the coefficients observed for GC/MS analysis in the EI mode are significantly different from those observed for the flame ionization detector reported elsewhere (*1*). Using this method less common monosaccharides could also be identified by their mass spectra, for example, 2-*O*-methyl-Fuc, Kdo, and disaccharides and trisaccharides derived from glycosaminoglycans (*12*) or from specific glycoproteins (see below).

An additional problem concerns monosaccharides phosphorylated in position 6 (such as Man-6-phosphate) because the *O*-phosphate group is quite stable to methanolysis. In fact, this bond was not essentially cleaved during methanolysis, but during acylation with HFBAA. As previously reported (*13*), after methanolysis of yeast lipo-phosphomannans and formation of HFB derivatives using anhydrous conditions, a small amount of the phosphate-di-methyl-ester of Man was detected, showing the initial presence of Man-6-phosphate. When applying the present approach to compounds having this type of bonds, the *O*-phosphate ester were first partially trans-esterified by HFBA during the first acylation step. Therefore, after methanolysis followed by a second step of acylation with HFBAA, the *O*-phosphorylated compounds were quantitatively transformed into per-HFB-derivatives of *O*-methyl-glycosides. As mentioned above, the first acylation step with HFBAA also blocks the free amino group of GlcN found in GPI-anchors (GlcN−Ins), the *O*-glycosidic bond involving N(HFB)-GlcN being cleaved by methanolysis.

For quantitation of *O*-methyl-glycosides, the integration of the peaks was performed by measuring the area of the major anomer (except for the reducing GlcNAc and Xyl, for which the two peaks are considered) and by performing a correction taking into account both the proportion of this anomer and the specific molar response of each compound. The corrected areas were obtained by multiplying the reported areas of the major anomers with the following factors (*3*): Xyl: 1.955; Fuc: 2.312; GlcN (reducing GlcNAc residue): 1.850; Gal: 2.025; Man: 1.075; Glc: 1.482; GalNAc: 2.472; GlcNAc: 1.551; NeuAc (and other sialic acid derivatives): 1.000; disaccharides: 2.000; trisaccharides: 3.000. Since the Xcalibur software allows the areas of the selected peaks to be copied into Microsoft Excel, a preexisting coefficient table immediately provides the corrected areas as well as the molar ratio of the various monosaccharides.

(c) Amino Acids. The HFB derivatives of the isoamyl-esters of the amino acids (Figure 2) are all separated from each other (Figure 3c) as well as from the monosaccharide constituents (*O*-isoamyl-glycosides) remaining after acid hydrolysis. To solve the problem of the actual structure of each amino acid derivative, analyses by mass spectrometry in the EI and CI mode of ionization were performed. All amino acid derivatives provided an intense pseudo-molecular ion at $m/z = [M + NH_4]^+$ in the CI mode and a minor pseudo-molecular ion at $m/z = [M + H]^+$, without significant fragmentations (not shown). Nevertheless, analyses were routinely performed in the electron impact mode and, indeed,

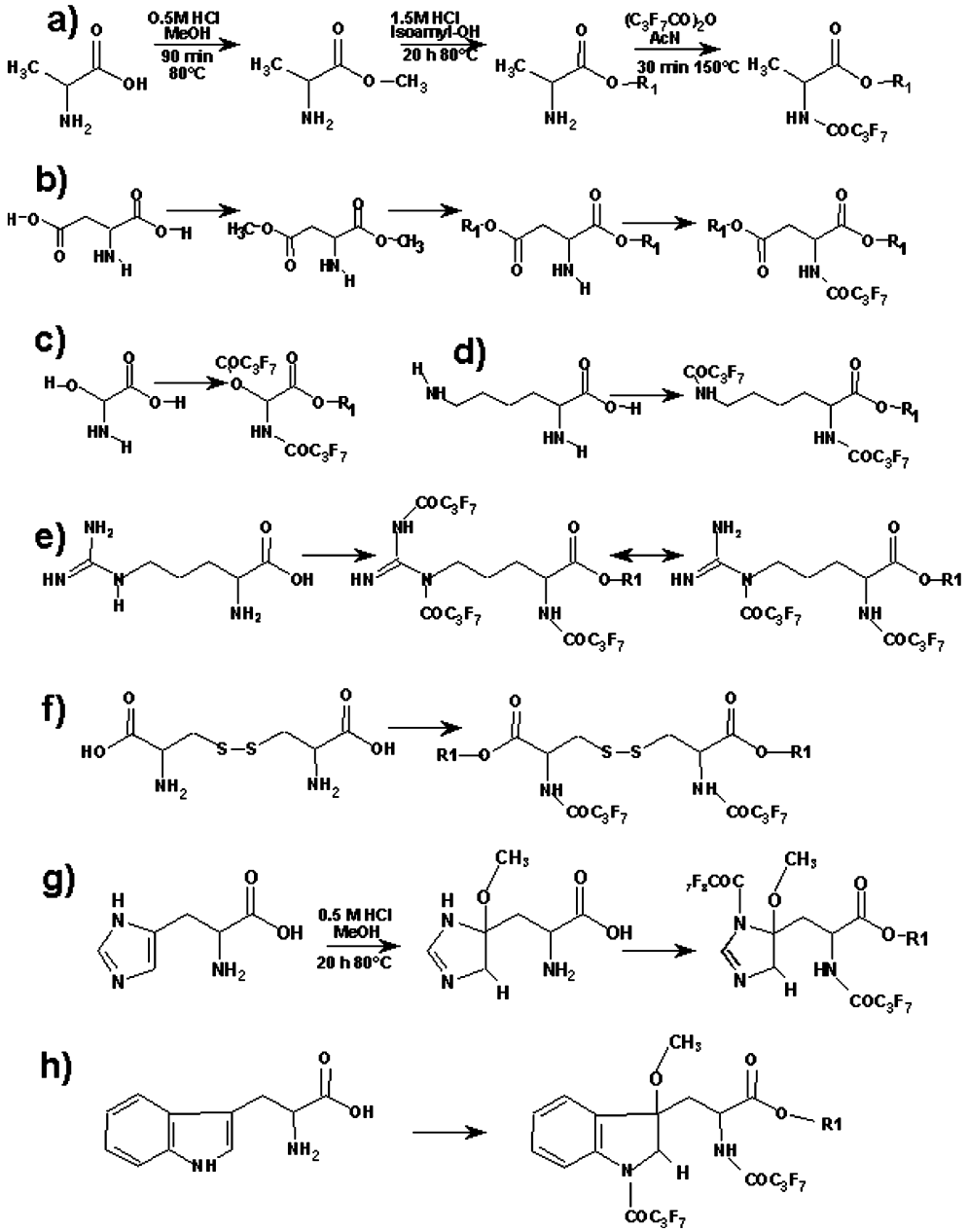

FIGURE 2: Flowchart of the derivatization procedure and proposed structures for the volatile derivatives of representative amino acids. (a, b) Flowchart of the chemical reactions producing volatile derivatives of Ala and Asp. (c) Example (Ser) of the products obtained from amino acids having an alcohol, phenol or thiol group. (d) Example (Lys) of the products obtained from amino acids having an additional amino group. (e) Structures of the volatile derivatives of Arg. The major and symmetrical peak corresponds to the fixation of three HFB groups. The other trailing peak corresponds to an unsubstituted guanidinium group. (f) Structure of the derivative of Cys_2. (g) Structure of the derivative of His. (h) Structure of the derivative of Trp. These structures were verified through the interpretation of their EI spectra and the mass of the derivatives was verified in the CI mode of ionization. Furthermore, the completeness of the trans-esterification procedure was verified using the specific search of ions corresponding to HFB-derivatives of methyl-esters as described elsewhere (*3*). These analyses indicated that methyl esters represented less than $1/10^6$ of the quantity of isoamyl-esters. The position of the $O-CH_3$ group added during acid-catalyzed methanolysis on His and Trp is still speculative. That of Trp (position 3 of the indol group was suggested by the fact that during hydrolysis, the major byproduct of Trp degradation was β-3-oxindolalanine (*14*, *15*).

the EI mass spectra of HFB derivatives of the isoamyl-ester of amino acids showed many interesting features (Table 1, Figures 4 and 5). The basic ion was generally not derived from HFB derivatives (69, 119, and 169) as observed for the *O*-methyl-glycoside derivatives analyzed in step 2, but the ion at $m/z = 71$ $[CH_2CH_2CH(CH_3)CH_3]^+$ derived from the isoamyl-alcohol. Except in a few cases (Cys_2 and Arg and the modified His and Trp residues (see below)), the molecular ion was of very low intensity. In contrast intense ions were generally observed at $m/z = M - 115$ ($M - COOCH_2CH_2CH(CH_3)CH_3$). Specific ions due to the presence of desaturated cyclical compounds were important for the identification of Phe and Tyr (ion at $m/z = 90$ and 91) and of His (ion at $m/z = 81$ and 82). The precise analysis of the fragmentation patterns of each peak allowed the unambiguous identification of each compound, for example an easy discrimination between Leu, Ile, and Nle (Figure 4). Even the nature of contaminants present in commercially available amino acid standard mixtures present at the picogram level of injected material could be identified.

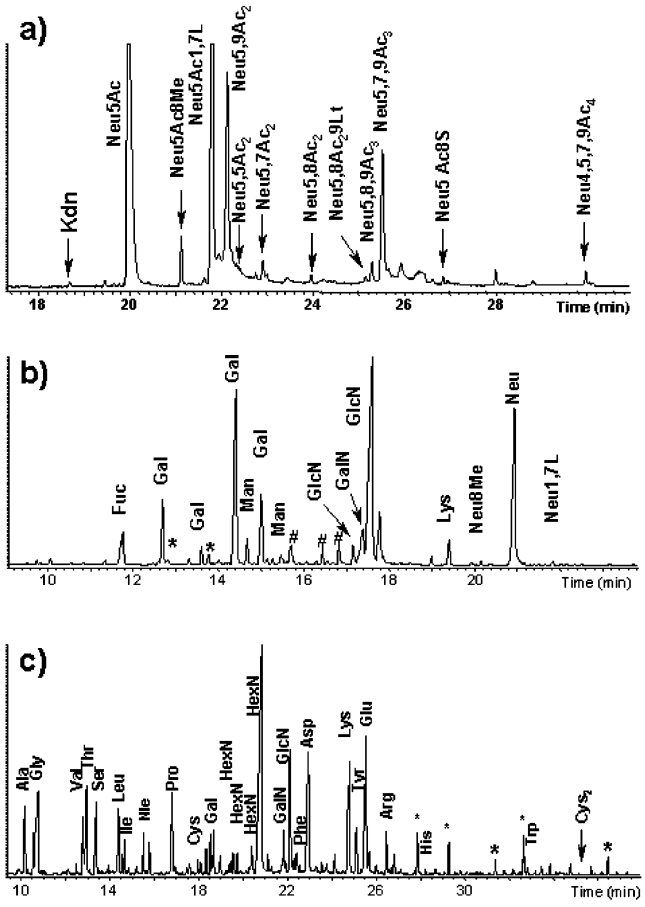

FIGURE 3: Chromatogram of the GC/MS (EI mode of ionization) of the constituents of glycoproteins from the human rectum as HFB derivatives. (a) Analysis of sialic acids; (b) Analysis of *O*-methyl glycosides; (c) analysis of amino acids. The nomenclature of the different sialic acids was according to ref 12.

Repetitive analyses of amino acid standard mixtures were used to assess the relative molar responses RMR) of the various amino acids relative to the internal standard norleucine (Nle) as shown in Table 1.

When analyzing amino acid standards, Cys was entirely recovered as the cystine derivative (Cys_2), eluting as a symmetrical peak (rRt = 2.273). No trace of Cys was detected and the oxidation of thiol groups during the derivatization procedure was also observed when the analyzing standards containing both Cys and homocysteine in equimolar amounts. The GC/MS analysis indicated that total transformation of these compounds occurred providing a mixture of Cys_2, $homoCys_2$ and the hybrid Cys-homoCys compound with molecular ions at *m*/*z* = 772, 800, and 786, respectively. The behavior of Cys in hydrolysates was different, especially when hydrolysis was not performed under vacuum. Under these conditions, Cys was oxidized to various extents to Cys_2 and $CysSO_3H$ (cysteic acid). The latter compound was recovered as a symmetrical peak of the HFB derivative of its di-isoamyl-ester, the sulfonic acid group being esterified with isoamyl-alcohol. *S*-carboxy-methyl-cysteine (SCCys) obtained after reduction and alkylation with iodoacetamide was obtained as the HFB derivative of its di-isoamyl-ester eluted just after the Asp derivative (rRt = 1.495; Table 1; Figure 5). After hydrolysis, a very small part of this compound (around 1%) was recovered as its sulfone derivative, similarly to what happened for Met (see below).

Met appeared as three different symmetrical peaks corresponding to Met, $MetSO_2$, and MetSO, indicating partial oxidation during the derivatization procedure. However, Met was always the major compound (Table 1; Figure 4).

Arg was recovered as two peaks corresponding to the tri-HFB derivative (symmetrical peak at rRt = 1.709) and as the di-HFB derivative (a severely trailing peak at rRt = 2.214). The presence of a HFB group on one of the two nitrogen atoms of the guanidinium group was sufficient to decrease the interaction of the free nitrogen atom with the column. During analyses of amino acid standard mixtures and glycoprotein hydrolysates, the proportion the two peaks was constant and quantitation of Arg could thus be performed by integrating only the first peak.

(d) Trp and His. The behavior of Trp during the sequential analysis procedure was of particular interest since this amino acid was considered to be destroyed during conventional acid hydrolysis, the major byproduct being beta-3-oxindolyl-alanine (*14*−*15*). However, the derivative of this compound or of Trp, was never detected during the amino acid analysis step. As shown in Figure 2, when submitted to acid-catalyzed methanolysis (step 2), Trp is quantitatively transformed into a derivative with a molecular ion at *m*/*z* = 642, indicating the addition of a methanol group (M = 32) as shown by GC/MS of its HFB derivative. The addition occurred on the double bond between the $C_{(2)}$ and $C_{(3)}$ carbon atoms of the indol ring, as suggested by a series of ions at *m*/*z* = 358 and 371 corresponding to the modified indol ring (Figure 5). The addition of a very stable methoxy group on the $C_{(2)}/C_{(3)}$ carbon atoms of the ring and the rupture of the delocalization of the double bonds of the indol cycle rendered the molecule much more stable to the strong acid conditions of step 3 and provided a true secondary amino group on the indol ring, that could be easily derivatized with HFBAA. The stability of the derivative was evidenced by the almost complete recovery of Trp as the di-HFB derivative of the isoamyl-ester of the methyl-ether derivative after acid hydrolysis. This compound gave rise to a molecular ion at *m*/*z* = 698 (exact mass = 698.448), i.e., a difference of 56 *amu* (i.e., that between isoamyl- and methyl-groups) relative to the methyl-ester derivative produced during acid-catalyzed methanolysis. From the analyses of standard mixtures, an rMR value of 0.741 relative to Nle was obtained and this value was unaffected by acid hydrolysis, indicating that no degradation of this Trp derivative occurred.

Depending on the experimental conditions, His gave rise to two types of derivatives. During perfectly anhydrous conditions of methanolysis (anhydrous reagents and analyzing low amounts of glycoproteins, i.e., less than 50 μg of material), a single symmetrical peak was obtained (rRt = 1.739) with a molecular ion at *m*/*z* = 649. On the basis of its mass and fragmentation pattern (Figure 5), this compound was deduced to result from the addition of a methanol group on one of the double bond of the imidazol ring as for Trp. This addition allowed the suppression of the delocalization of the double bonds of the initial ring structure, and thus the appearance of a true secondary amino group that was quantitatively derivatized with HFBAA. During the subsequent acid hydrolysis step, this compound was quantitatively recovered. When using an inadequate glycoprotein/methanolysis reagent ratio (more than 100 μg of glycoprotein in 500 μL of reagent), this peak was absent and replaced by a

Table 1: Retention Times (Rt), Mass, Relative Retention Times to the Internal Standard Nle (rRt), Reporter Ions, Percent Distribution of the Various Peaks (%), and Relative Molar Responses (rMR) to Nle of Amino Acid and Hexosamine Derivatives of the Isoamyl Ester (*O*-Methyl-glycosides) of HFB Derivatives

aa	Rt	mass		reporter ions	%	RMR
Ala	10.03	355	0.6526	286−268−**241**−**240**−220−192−169−71−55	100.00	0.795
Gly	10.56	341	0.6871	272−254−**227**−**226**−206−178−169−71−55	100.00	0.770
Val	12.66	383	0.8237	341−314−296−**269**−**268**−254−226−214−169−100−71−55	100.00	0.757
Thr	12.75	581	0.8295	512−466−368−298−280−**253**−**252**−241−238−213−197−169−71−70−57−55	100.00	1.077
Ser	13.20	567	0.8588	498−452−284-**239**−**238**−210−169−119−100−71−70−55	100.00	0.966
Leu	14.22	397	0.9440	341−**283**−**282**−266−253−**240**−226−214−169−114−71−70−69−55	100.00	0.873
Ile	14.51	397	0.9252	341−**283**−**282**−271−**253**−240−226−214−184−169−114−71−70−69−57−55	100.00	0.946
Nle	15.37	397	1.000	341−309−**283**−**282**−240−**226**−**214**−169−114−71−70−69−55	100.00	1.000
Pro	16.62	381	1.0813	381−312−293−**267**−**266**−239−198−169−71−70−69−55	100.00	0.729
Met[a]	19.81	415	1.2889	**415**−**341**−298−279−**271**−**266**−**253**−**252**−238−225−131−169−75−71−70−61	97.87	0.557
Phe	21.97	431	1.4294	431−316−**218**−**149**−**148**−131−119−**103**−**92**−**91**−71−70−55	100.00	1.451
Asp	22.75	469	1.4802	454−400−354−312−284−266−**239**−214−169−141−99−72−71−55	100.00	0.934
Lys	24.51	608	1.5947	608−539−**520**−493−473−**281**−**280**−**226**−214−169−82−71−70−67−55	100.00	1.492
Tyr	24.90	643	1.620	643−628−574−554−**528**−508−**430**−**360**−**303**−275−169−107−90−71−70	100.00	1.437
Glu	25.34	483	1.6487	483−414−396−368−326−307−**298**−**280**−**252**−239−169−85−71−70−55	100.00	1.102
Arg[b]	26.27	832	1.7092	**832**−813−763−717−**663**−593−**504**−**492**−478−266−214−169−100−71−70	64.56	0.213
Arg[c]	34.03	636	2.2141	636−621−591−567−521−**467**−451−397−296−282−266−169−138−85−71	33.86	n.d.
His#[d]	26.74	649	1.739	**649**−**578**−433−408−383−322−**81**−71−69	100.00	0.712
His	28.19	421	1.8341	421−**306**−288−252−208−169−139−121−109−94−82−**81**−71−69−55	100.00	0.177
Trp#[e]	32.66	698	2.125	**698**−629−**611**−584−486−398−**371**−359−**303**−275−240−103−71	100.00	0.741
Cys_2	34.94	772	2.2733	**772**−657−587−559−**354**−**284**−254−**238**−171−169−**103**−71−70−55	100.00	0.322
GlcNp	20.42	1033	1.3288	946−926−818−731−**703**−702−606−518−498−490−489−276−81−72	75.17	2.217
GlcNp	21.72	1033	1.4129	946−926−818−731−**703**−702−606−518−498−490−489−276−81−72	24.83	2.217
GalNf	19.20	1033	1.2492	946−926−818−731−**703**−702−606−580−518−498−490−489−276−81−72	12.87	2.217
GalNf	20.10	1033	1.3074	946−926−818−731−**703**−702−606−580−518−498−490−489−276−81−72	14.06	2.217
GalNp	20.42	1033	1.3288	946−926−818−731−**703**−702−606−518−498−490−489−276−81−72	56.91	2.217
GalNp	21.41	1033	1.3929	946−926−818−731−**703**−702−606−518−498−490−489−276−81−72	16.16	2.217
Hypro	18.58	593	1.209	593−574−524−504−**478**−380−310−**265**−**264**−169−70−69−67−55	100.00	n.d.
HyLys	25.96	820	1.689	820−805−606−591−564−**491**−276−263−169−101−71	100.00	n.d.
$MetSO_2$[a]	23.97	447	1.559	447−**419**−**368**−349−**298**−284−270−**252**−240−197−169−**93**−71−55	2.01	n.d.
MetSO[a]	24.80	431	1.613	431−415−368−298−**252**−240−195−169−125−100−84−71−61−55	0.12	n.d.
Orn[b]	22.19	594	1.443	594−525−**506**−479−394−363−281−**267**−**266**−226−185−71	1.58	n.d.
$CysSO_3$	31.77	505	2.067	**506**−**436**−419−**391**−358−306−252−**194**−**177**−165−136−107−71−69		n.d.
SCCys	22.90	515	1.495	**444**−**374**−356−329−239−177−169−159−**115**−103−71	100.00	0.644

[a] The GC/MS analyses in the EI and CI mode of ionization of Met derivatives indicate the presence of traces of MetSO and $MetSO_2$, possibly resulting from oxidation of Met during the formation of derivatives. rMR of Met is given for the area of the major peak of Met taking into account the artifactual production of MetSO and $MetSO_2$ during the derivatization procedure. [b,c] Two derivatives of Arg were obtained corresponding to the tri-HFB (symmetrical peak) and the di-HFB derivative of Arg-isoamyl-ester (trailing peak). Traces of ornithine (Orn) and hydroxylysine were found in the commercial standard and in the hydrolysis product. For Arg, the rMR is given relative to the symmetrical peak at rRt = 1.709. The rMR of hexosamines were calculated taking into account all peaks of hexosamine derivatives. [d] His#: di-HFB derivative of the isoamyl-ester of the derivative of His resulting from the addition of a CH_3OH group to the imidazol ring during acid-catalyzed methanolysis. [e] Trp# corresponds to the modified Trp during acid-catalyzed methanolysis.

severely trailing one (rRt = 1.834) with a molecular ion at $m/z = 421$ corresponding to the nonderivatized imidazol ring (Table 1). Due to the severe trailing and the presence of contaminants, the determination of its area must be performed on the reconstituted chromatogram for the ion at $m/z = 169$ and relating this value to that of Nle in the same reconstituted chromatogram. The importance of the protein−glycoprotein ratio to the reagent is probably not explained by a significant production of water during the methanolysis cleavage of glycosidic and acetamido bonds during the methanolysis step, but to a decreased concentration of the HCl catalyst by protonation of the polypeptide basic groups in the polypeptide. This could explain the all or nothing pattern for His derivatives. However, it should be pointed out (see below) that the approach described here provides optimal results only at sub-nanomolar concentrations of sample and that excess sample will affect the reliability of all steps in a negative way. When larger quantities of sample are used, the amount of reagents must be increased in proportion.

Problems Encountered in Glycoprotein Analyses. Hexosamines are known to be resistant to the acid hydrolysis conditions used for cleaving peptide bonds, whereas other monosaccharides are to a large part destroyed. However, using our procedure, the neutral monosaccharides are (as HFB derivatives of *O*-methyl-glycosides) initially more stable than the corresponding underivatized monosaccharides and, consequently, present as significant peaks on the GC/MS chromatogram of the amino acid analysis. As expected, Glc gave two peaks corresponding to the α and β anomers, as did Man, whereas Gal and Fuc gave four peaks corresponding to the two pyranic anomers and the two furanic ones. However, none of these hexose and deoxy-hexose peaks interfered with those of the amino acids. The hexosamine derivatives were also completely separated from the amino acid peaks, and thus did not interfere with the determination of the amino acid composition. As expected, the derivative of GalNAc gave four peaks corresponding to the anomers of the pyranic and furanic forms, whereas GlcNAc gave two peaks corresponding to the anomers of the pyranic form. It is emphasized that the EI spectra allowed us to unambiguously distinguish between pyranic and furanic forms of the HFB derivatives of the *O*-isoamyl-glycosides because of the

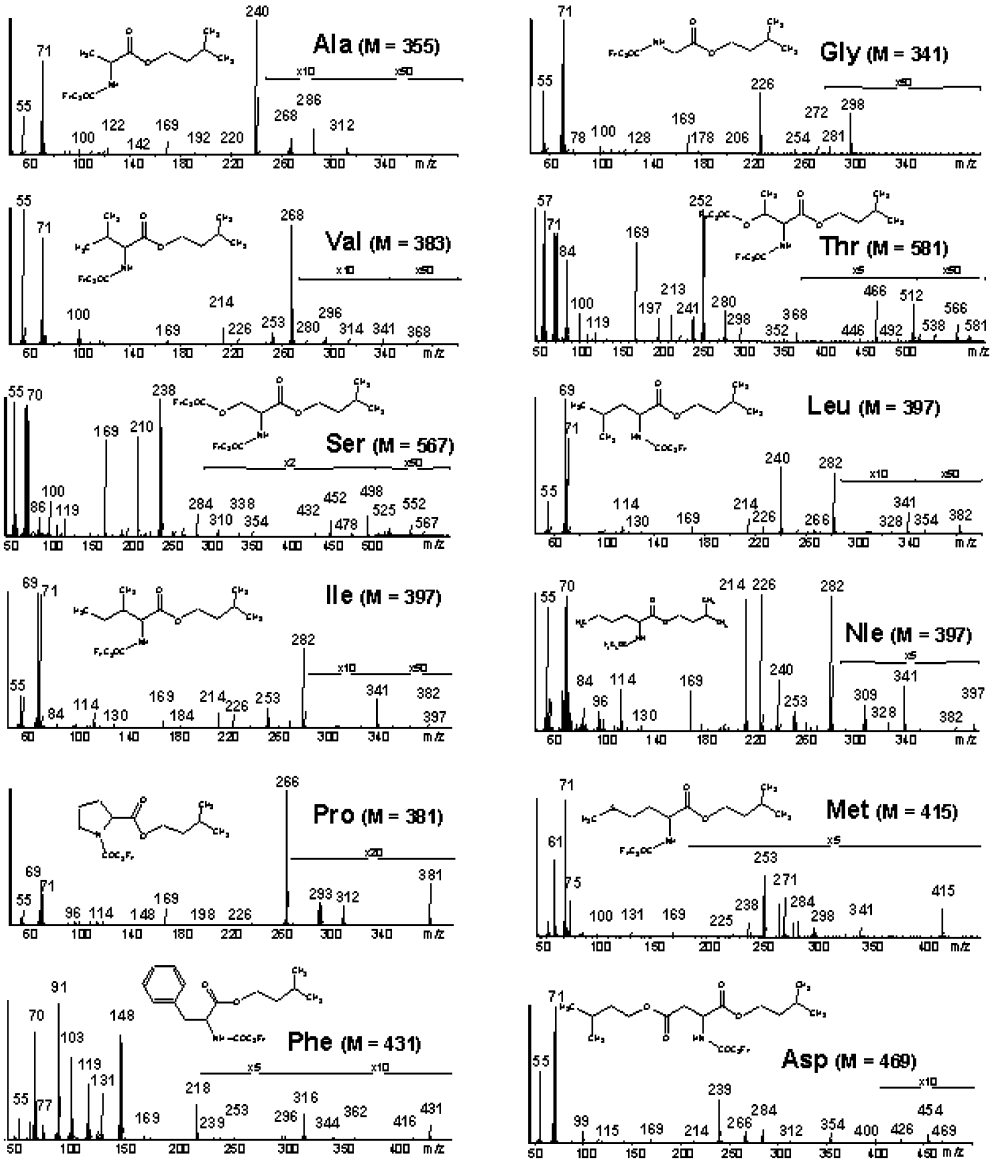

FIGURE 4: Mass spectra (EI mode of ionization) of the HFB derivatives of the isoamyl-esters of amino acids (Ala, Gly, Val, Thr, Ser, Leu, Ile, Nle, Pro, Met, Phe, Asp).

intense E1 ion for the furanic forms of the *O*-isoamyl-glycoside of GalN ($m/z = 580$; the equivalent of the E1 ion at $m/z = 525$ (*1*) for the *O*-methyl glycoside of Gal and at $m/z = 524$ for the furanic forms of the *O*-methyl-glycoside of GalN). These E1 ions were similar to those described for *O*-methyl glycosides (*1*) but with the mass increment of 56 *amu* (i.e. the difference between methyl- and isoamyl-groups). Unfortunately, the major peaks from GalNAc and GlcNAc had exactly the same Rt and, consequently, the ratio between GalNAc and GlcNAc could not be determined directly using their major peaks. Nevertheless, since the proportion of the different anomers is constant (Table 1), a single calculation based on the quantitation of the peak at rRt = 1.393 of GalNAc and that at rRt = 1.329 of the mixture of GalNAc and GlcNAc, representing 16.16 and 75.17% of each hexosamine, respectively, allowed us to obtain this ratio: $A_{GalNAc} = A_{rRt1.393}/0.1616$ and $A_{GlcNAc} = (A_{rRt1.393} - A_{GalNAc} \times 0.5691)/0.7517$. In fact, this ratio could be obtained directly in the second step of the method, i.e., when analyzing the monosaccharide composition, provided that the peaks corresponding to the Asn-linked glucosamine were also included. The two calculations gave identical results (Table 2), indicating that hexosamines were not significantly destroyed during acid hydrolysis of proteins.

The instability of other monosaccharides during acid-hydrolysis gave rise to a very large number of peaks. Almost all degradation products of hexoses had high rRt (rRt = 1.654, 1.798, 1.960, 2.122, and 2.318 (Figure 3c), respectively, for the major degradation products) and, therefore, did not interfere with the amino acid peaks. Nevertheless, it was surprising to recover relatively high amounts of hexoses (and also Fuc) after acid hydrolysis known to strongly destroy these compounds. The reason hexoses and to a lesser extent Fuc are preserved is explained by the fact that the monosaccharides occur as HFB derivatives of their *O*-methyl-glycosides due to the previous step of the analysis. Although this was not investigated in details, the resistance may involve a cooperative stabilization of the monosaccharides during acid hydrolysis by the blockage of the $C_{(1)}$ environment as an *O*-methyl-glycoside and by a general protection of the monosaccharide molecule by the HFB derivatives, the HFB bond to hydroxyl and amino groups being very resistant to

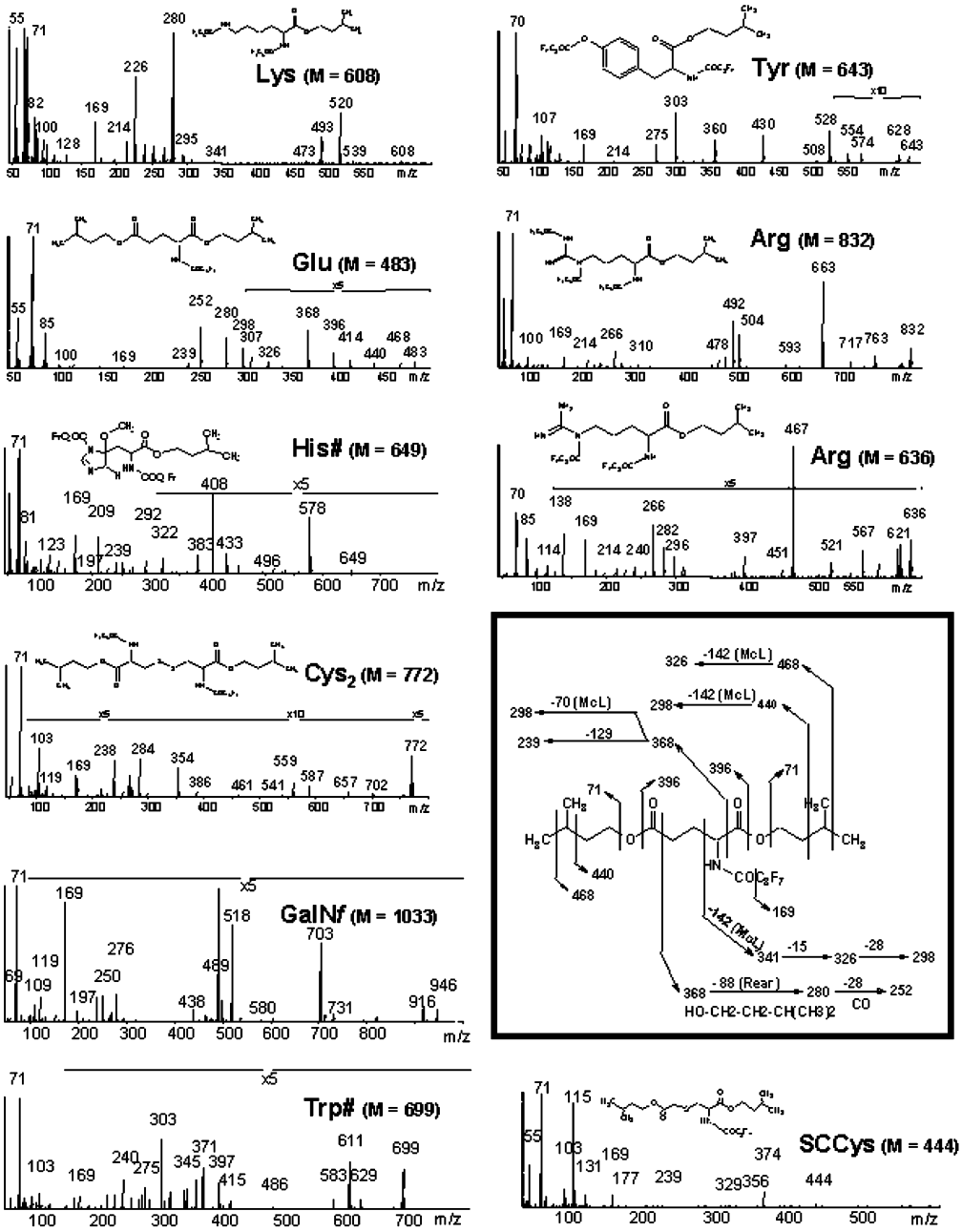

FIGURE 5: Mass spectra (EI mode of ionization) of the HFB derivatives of the isoamyl-esters of amino acids (Lys, Tyr, Glu, Arg, His# (transformed by methanolysis) and Cys_2, of an *O*-isoamyl-glycosides of a furanic form of galactosamine GalN*f*, of Thr derivative of Trp# and of *S*-carboxy-methyl-Cys (SCCys)). Inset: detailed fragmentation mechanism of the derivative of Glu. Arg (M = 832) corresponds to the tri-HFB derivative of the isoamyl-ester of Arg, the symmetrical peak used for integration. Arg (M = 636) corresponds to the trailing peak of Arg. Inset: the intense ion at $m/z = 307$ is due to the concerted elimination 2 isoamyl alcohol groups ($M - 2 \times 88$) by the formation of two 6-atom rings (Rear). This situation was only observed for the Glu derivative, the other amino acid derivatives (such as Asp) showing a single low of isoamyl alcohol. McL = Mac Lafferty mechanisms of fragmentation.

acidic conditions (*1*). Nevertheless, neutral sugars were not quantitatively recovered after acid hydrolysis in contrast with hexosamines.

Complete Analysis of Glycoproteins from the Human Rectum. As shown in Figure 3a, sialic acids from human rectum showed a very large diversity (in the order of Rt: Kdn, Neu5Ac, Neu5Ac8Me, Neu5Ac1,7L, Neu5,9Ac_2, Neu, Neu4,5Ac_2, Neu5,7Ac_2, Neu5,8Ac_2, Neu5,8$Ac_2$9Lt, Neu5,7,-9Ac_3, Neu5Ac8S, and Neu4,5,7,9Ac_4 were identified). The analysis of different human samples displayed significant differences; however, repetitive analyses of the same sample provided reproducible results. As previously described (*4*), the HFB derivatives of the methyl-esters of sialic acids were stable with time provided that they were kept in dried acetonitrile in the presence of HFBAA. The biological significance of the pronounced sialic acid diversity of colonic mucins remains to be elucidated. However, the approach described here will allow the analysis of the small amounts of material obtained, for example, from clinical biopsies and, consequently, this feature may be studied using samples obtained from different anatomical locations and in various clinical conditions.

The subsequent monosaccharide composition performed on the same material (Figure 3b) showed the presence of Fuc, Gal, Man, GalN, GlcN, and sialic acid as major constituents. Detailed analyses of minor compounds indicated

Table 2: Composition of the Human Uromodulin and of the Human MUC2 Mucin[a]

sialic	MUC2 (%)	uro (%)	sugar	MUC2 (%)	uro (%)		MUC2 (%)	theo (%)	uro (%)	theo (%)
Kdn	0.38	0.00	xyl	0.92	0.74	Ala	4.36	2.23	7.57	6.67
Neu5Ac	47.57	59.87	fuc	9.12	9.04	Gly	14.09	5.45	11.20	8.29
Neu5Ac8Me	1.01	1.62	glcN	3.31	3.10	Val	4.90	5.27	6.00	6.67
Neu5Ac9Lt	0.00	0.00	Gal	14.20	17.51	Thr	21.29	35.08	5.77	7.80
Neu5Ac1,7L	7.77	35.12	Man	2.12	12.24	Ser	8.12	4.69	6.52	8.62
Neu5,9Ac_2	26.56	0.96	Glc	3.13	5.08	Leu	3.38	3.14	7.29	8.62
Neu	0.21	0.30	GalNAc	31.04	9.08	Ile	1.64	3.66	1.72	2.60
Neu4,5Ac_2	0.20	0.00	GlcNAc	15.54	18.60	Pro	13.16	15.85	6.79	4.56
Neu5,7Ac_2	3.29	0.00	Neu8Me	0.59	0.45	Met	0.65	0.66	0.76	1.95
Neu5,8Ac_2	0.00	0.00	Neu	13.92	11.49	Phe	3.57	1.49	3.72	3.26
Neu5,8,9Ac_3	3.43	0.00	Neu1,7L	0.88	1.22	Asp	6.82	4.61	12.25	10.24
Neu5,8$Ac_2$9Lt	3.43	1.08	disacch	3.84	0.71	Lys	1.75	2.07	4.31	2.60
Neu5,7,9Ac_3	4.19	0.00	disacch	1.38	4.20	Tyr	1.51	1.71	4.29	3.58
Neu5Gc	0.00	0.00	disacch	0.00	2.48	Glu	7.91	6.16	8.71	7.80
Neu5Ac8S	0.93	1.09	disacch	0.00	0.34	Arg	1.11	1.67	5.17	4.88
Neu4,5,7,9Ac_4	1.04	0.00	trisacch	0.00	2.03	His	1.13	1.49	2.10	2.44
Other	0.00	0.00	trisacch	0.00	1.70	Trp	0.77	0.62	1.31	1.62
						Cys*	3.86	4.15	5.85	7.80

[a] Data were obtained starting from the same sample using three GC/MS steps in the same chromatographic system after different procedures of cleavage. Data are expressed as % molar ratio of the individual constituents in each category. For Uro glycoprotein, the proportion of GalNAc and GlcNAc obtained after methanolysis (32.00 and 67.20%, respectively) was identical to that obtained after acid hydrolysis (31.81 and 68.19%, respectively), indicating the absence of degradation of these constituents during acid hydrolysis of protein. The recovery of Neu5Ac8Me after methanolysis (1.70% of the total sialic acids) was in the same range as that obtained in the sialic acid analysis step (1.62%), indicating that the 8-methyl group was stable to methanolysis. The composition indicated that glycans represent 26% of the total weight of the molecule. In these samples, a relatively high number of glycosidic bonds involving hexosamines were recovered as di- and trisaccharides, a mechanism never observed in "classical glycoproteins". For MUC2, the sialic acid composition was very heterogeneous; however, no traces of Neu5Gc were detected in the two glycoproteins. The composition of MUC2 indicated that sugars represent 65% of the weight of the molecule. Theo = theoretical value deduced from the cDNA sequencing (23,24). Cys* in MUC2 was entirely recovered as the derivative of *S*-carboxymethyl-Cys.

also the initial presence of Neu5Ac8Me and although the acetamido group of Neu5Ac8Me was lost during methanolysis, the 8-methyl group was still being resistant. The per-HFB derivative of the *O*-methyl-glycoside derived from Neu5Ac8Me was different from that of Neu5Ac (methyl group in position 8 instead of a HFB group), and this peak was identified by its mass spectrum. Its proportion relative to the sum of other sialic acids (*O*-acylated sialic acids gave the same derivative as Neu5Ac after methanolysis) was very close to that expected from the sialic acid analysis. A second point was the presence of a significant peak corresponding to the derivative of Neu5Ac1,7L. As previously reported (*4*, *16*), this lactone is relatively stable during alkaline and acidic conditions and, despite a destruction of more than 60%, could be recovered as a peak different from that of other Neu5Ac derivatives after acid-catalyzed methanolysis. Although the proportion may vary between samples, the presence of a significant amount of this compounds in methanolysis products indicated its presence in the initial sample.

The GC/MS profile of the HCl hydrolysis products (Figure 3c) showed the absence of interferences between hexose and hexosamine derivatives (and degradation products of other monosaccharides) with the amino acid derivatives thus providing a straightforward identification of the former and other compounds through their EI mass spectra.

Complete Analysis of Purified Glycoproteins. Although the approach efficiently identified all sialic acids, monosaccharides, and amino acids in unpurified materials, the absence of data on the actual composition of this material did not allow the demonstration of the validity of the method from the quantitative point of view. Therefore, two purified glycoproteins, the human mucin MUC2 mucin (possessing essentially O-linked glycans) and the uromodulin (Uro) glycoproteins (possessing essentially N-linked ones) were analyzed because the polypeptide sequence and the carbohydrate composition of these molecules have been determined before. The results are summarized in Table 2.

MUC2 displayed a very large diversity of sialic acids and besides Neu5Ac, which is the prominent constituent, *O*-acetylated derivatives of Neu5Ac were major species identified. Traces of Kdn and of Neu4,5Ac_2 were also detected and these compounds have recently been identified by using the same technique in human red blood cells (*16*). A major finding concerning the Uro glycoprotein was that 35% of the sialic acids were present as the intramolecular 1,7-lactone of Neu5Ac first detected in tissues by Zanetta et al. (*4*). This compound has previously been overlooked because all other methods relied on the initial purification of the released sialic acids by ion-exchange chromatography where the uncharged lactone was not recovered. The lactone was identified because it was the only sialic acid derivative that was recovered omitting the methyl-esterification step with diazomethane after mild acid hydrolysis. Of particular importance in this context is that Neu5Ac1,7L is the specific ligand of human IL-4 (*17*) and that the isolated compound was able to inhibit IL-4 changes in phosphorylations on resting human lymphocytes (Cebo et al., in preparation). This observation is in keeping with previous works (*18*) showing that urine glycoproteins from pregnant women specifically bound IL-4 in a carbohydrate-dependent way involving sialic acids. The data presented here support this idea and suggest that the ligand of IL-4 in urine glycoproteins of pregnant women is actually this lactone. Traces of this compound were still detectable after methanolysis as a small peak eluting after the derivative of Neu giving an intense ion at $m/z = 833$. The present method also allowed us to verify that Neu5Ac8Me is resistant to acid-catalyzed methanolysis since this compound was quantitatively recovered as a compound eluting before the derivative of Neu5Ac (Table 2). The overall composition of MUC2 and of Uro obtained here are in very

good agreement with data previously reported in the literature (Table 2), in particular, concerning the ratios between the various monosaccharide and the carbohydrate content of the molecules.

A special feature of the analyses of the two glycoproteins as compared with classical glycoproteins containing N- and/or O-glycans was the presence of compounds eluting as disaccharides and trisaccharides (*10*). For the Uro glycoprotein, six peaks were identified with rRt 1.647, 1.675, 1.721, and 1.817, 1.973, and 2.054, respectively, the first four corresponding to disaccharides and the later to trisaccharides. Detailed MS analysis of the fragmentation spectra indicated that the two first (and major) peaks contained GalNAc in the nonreducing position (absence of the E1 ion at $m/z = 508$), whereas the third and fourth peaks had GlcNAc in the nonreducing end. No traces of the characteristic ions of hexoses or uronic acids (*10*) were found and these compounds are likely to be formed by hexosamine residues only. The reasons these disaccharides are still present after methanolysis is still obscure. Possibly, the N-acetyl group of *N*-acetyl-hexosamines is lost during mild acid hydrolysis conditions used in the preceding step liberating sialic acids and the exposure of a free NH_2 group, which would prohibit acid-catalyzed cleavage because of the shielding effect of its protonated form (*12*). This hypothesis is, however, unlikely because the NH_2 group would be acylated by HFBAA. This point was verified by analyzing glycosylphosphatidylinositols and it was observed that the HFB substituent of the amino group is cleaved by acid-catalyzed methanolysis after cleavage of the glycosidic bond between GlcN and inositol. In fact, the mass spectra of these disaccharides showing the presence of only hexosamine derivatives also contradicted the previous hypothesis and rather suggested that these disaccharides resulted from GalNAc−GlcNAc and GlcNAc−GalNAc disaccharides present in the Uro glycoprotein, the HexNAc−HexNac bonds being particularly resistant to acid-catalyzed methanolysis. Considering the present knowledge of the structure of N- and O-glycans of Uro (*19*−*21*), it was suggested that the two anomers having GalNAc in a nonreducing position belong to N-glycans (*20*−*21*), whereas the two anomers having GlcNAc in the nonreducing position result from core 2 O-glycans that are particularly abundant in uromodulin (*20*).

A major consideration of this approach is how well the results of the amino acid analysis relate to those obtained using "classical techniques", considering the fact that this information is gathered "downstream" to the same GC/MS apparatus, the same chromatographic conditions, and almost the same reagents (except the trans-esterification reagents). The amino acid composition of the two purified glycoproteins obtained using classical conditions of protein hydrolysis (Table 2) showed convergent results with the theoretical values. Comparisons of the amino acid molar ratios with those deduced from the amino acid sequence of the two glycoproteins indicated, as the major discrepancy is a lower recovery of Thr and Ile and the higher recovery of Gly. The low recovery of Ile may result from uncleaved sequences and that of Thr from a destruction of this amino acid considered as the most sensitive to acid hydrolysis (*22*). But, even the case of the amino acids causing problems in classical analyses, Arg, Cys, His, Met, and Trp, the determined rMR of these compounds allowed a good correlation with the classical techniques.

In our approach, a single internal standard was used, Nle added after the hydrolysis step. This appeared to be sufficient to calculate the contribution of sialic acids and monosaccharides to the glycoprotein. Indeed, the molar ratio of the different sialic acids was obtained in the first step of the analysis. The molar ratio of sialic acids relative to other monosaccharides and, especially, hexosamines was obtained in the second step of the analysis. The monosaccharide contribution of monosaccharides to the glycoprotein was deduced from the hexosamine content in the third step of the analysis. Due to the presence of the internal standard (Nle), not only the relative contribution of each type of constituent could be determined, but also their absolute amount (Table 2).

Problems Related to the Quantity of Material. GC/MS analyses of HFB derivatives presented significant advantages as compared to other derivatives, because HFB-derived ions (69, 119, and 169, corresponding to CF_3, CF_2CF_3, and CF_2-CF_2CF_3) are not frequently occurring in biological samples. As demonstrated in a previous paper (*3*), very complex GC/MS chromatograms of highly impure samples could be safely interpreted performing chromatogram reconstitution for the previous ions specific of HFB derivatives and therefore, the signal/background ratio of HFB derivative is always very high. The chromatogram reconstitution technique with specific ions of HFB eliminates virtually all peaks arising from liquid-phase leakage, from phthalates (use of plastic tubing) and analyses could thus be performed starting with very low amounts of sample. As reported before (*3*), optimal conditions of analysis are achieved when the detector response is not saturated, and this is usually obtained for quantities lower than 1 ng of each initial constituent injected. For example, starting from a mixture of 1 nmol of each amino acid, optimal GC/MS conditions are obtained injecting 1 μL of the derivatives over 200 μL of the sample, i.e., 5 pmol. Since monosaccharide derivatives have a higher RMR, analysis at the same level (or between 10 and 100 times lower quantities) can be performed with the same efficiency. Therefore, analyses must be performed starting from less than 10 μg of glycoproteins, and when a higher amount of starting material is used, reagent volumes must be increased in proportion and the sample adequately diluted before GC/MS analysis. Furthermore, consistent data for the monosaccharide analysis could be obtained from samples adsorbed onto PVDF membranes (as soon as a significant protein stain could be detected; approximately 1 μg of glycoprotein) allowing detailed chemical analysis to be performed on individual glycoprotein spots after 2D electrophoresis.

CONCLUSIONS

Using a single GC/MS apparatus, a single GC temperature program and a single detection mode, allowed us to obtain qualitative and quantitative analyses of sialic acids, the other monosaccharides as well as the amino acids of glycoproteins in the presence of a single internal standard, Nle. The same methodology was shown to provide a complete analysis of glycolipid constituents (*1*−*3*), including sialic acids, monosaccharides, FAMEs, and long-chains bases starting from the same sample and with the sample remaining in the same

reaction vessel. The approach could also be used for the constituents of sulfated proteoglycans with the modification that the methanolysis step must be performed in the presence of small amounts of barium acetate (*12*). Concerning glycosyl-phosphatidylinositol anchored glycoproteins, the sequential analysis presented here promotes blockage of the free amino group of the α-GlcN and thus the cleavage of the glycosidic bond between this residue and inositol. The electron impact ionization detection provides a systematic verification of each constituent (including di- and trisaccharides), a major advantage when compared with GC methods where the various constituents are only identified by their retention times. Finally, this method allows for the first time the quantitative determination of Trp in protein acid hydrolysates.

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