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Amino acid analysis by hydrophilic interaction chromatography coupled on-line to electrospray ionization mass spectrometry

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Summary. Collagens form a common family of triple-helical proteins classified in 21 types. This unique structure is further stabilized by specific hydroxylation of distinct lysyl and prolyl residues forming 5-hydroxylysine and hydroxyproline (Hyp) isomers, mostly 4-trans and 3-trans-Hyp. The molecular distribution of the Hyp-isomers among the different collagen types is still not well investigated, even though disturbances in the hydroxylation of collagens are likely to be involved in several diseases such as osteoporosis and autoimmune diseases. Here, a new approach to analyze underivatized amino acids by hydrophilic interaction chromatography (HILIC) coupled on-line to electrospray ionization mass spectrometry (ESI-MS) is reported. This method can separate all three studied Hyp-isomers, Ile, and Leu, which are all isobaric, allowing a direct qualitative and quantitative analysis of collagen hydrolysates. The sensitivity and specificity was increased by a neutral loss scan based on the loss of formic acid (46 u).

Keywords: Collagen types – ESI-MS – HILIC – Hydroxyproline – Hyp

Abbreviations: ESI, electrospray ionization; HILIC, hydrophilic interaction chromatography; Hyp, hydroxyproline; MS, mass spectrometry

Introduction

In the last decade several interface techniques to couple liquid chromatography to mass spectrometers have been commercialized to analyze nonvolatile compounds that cannot be analyzed by GC–MS. Depending on the physical and chemical properties of the compounds to be analyzed electrospray ionization (ESI) (Fenn et al., 1989), atmospheric pressure chemical ionization (APCI) (Horning et al., 1974), and more recently atmospheric pressure photoionization (APPI) (Raffaelli and Saba, 2003; Robb et al., 2000) are coupled on-line to the HPLC systems, whereas matrix-assisted laser desorption/ionization (MALDI) is coupled off-line (Gusev, 2000). However, not all chromatographic techniques are compatible with these ionization sources. Major restrictions are eluents that should contain only volatile solvents and salts. Thus, mostly reversed-phase techniques have been coupled to mass spectrometers, which also typically possess the highest resolution. Moreover, the low pH of most eluents in RPC, especially ion pair RPC (e.g., trifluoroacetic acid, TFA), assists the ionization process in positive ion-mode increasing the protonation efficiency of the solutes. Thus RPC–ESI-MS has become the method of choice in many research fields, especially in peptidomics and proteomics recently. More complex peptide mixtures can be analyzed by two-dimensional or three-dimensional LC techniques using ion-exchange and size-exclusion chromatography as initial separation techniques coupled on- or off-line to RPC–ESI-MS.

On the other hand, more polar compounds are not or only poorly separated due to weak retention on the reversed phase. Although this has been partially overcome by the use of graphitized carbon columns (Tornkvist et al., 2004), the standard procedure is pre-column derivatization. For example a large panel of derivatization reagents has been introduced for amino acids, such as o-phthaldialdehyde (OPA, Lee and Drescher, 1978), phenylisothiocyanate (PITC, Cohen et al., 1986), 9-fluorenylmethyl chloroformate (FMOC, Roturier et al., 1995; Einarsson et al., 1987), 4-(4-dimethylaminophenylazo(benzenesulfonyl chloride (DABSYL, Lin and Lai, 1980), N2-(5-fluoro-2,4-dinitrophenyl)-L-valine amide (FDVA, Brückner and Keller-Hoehl, 1990). Optimized gradients do not only allow separation of proteinogenic amino acids but also their modified analogs as well as amino acids present only in body fluids (Schwarz et al., 2005). The major advantages of these well established techniques are their good reproduc-

ibility, the relatively inexpensive equipment, and the high sensitivity in the low pmol-range that can be even further increased by fluorescence detection. However, these techniques are typically restricted to the simultaneous analysis of 30 or maybe 40 amino acids analyzed by a single well optimized gradient. Uncertainties arise when more complex mixtures are analyzed that could contain other amino acids or amino compounds with similar or identical retention times. This was partially overcome by coupling these techniques to mass spectrometers to identify coeluting compounds that are not isobaric. However, derivatization of the amino groups significantly decreases the ionization degree of amino acids in positive ion-mode reducing the sensitivity. Moreover, other underivatized impurities present in the sample or the reagents that are not seen in the UV trace, can produce intense signals in the mass spectrum.

Alternatively, polar compounds can be separated by hydrophilic interaction chromatography (HILIC, Alpert, 1990). Two gradient techniques have been described to elute polar compounds in HILIC. In the most common technique polar compounds are eluted from an amide, hydroxyl, cyano or amino-based stationary phase by reducing the organic content of the eluent. Typical gradients start at 80 to 90% aqueous acetonitrile and reduce the organic content to 60 to 40%. The retention times of the solutes increase with their hydrophilictiy. As these solvents contain only volatile solvents and salts at low concentrations, they can be effectively coupled to ESI-MS allowing for example the sensitive detection of underivatized amino acids that have only very low extinction coefficients in the accessible UV range until 210 nm. Alternatively, HILIC mode is also obtained on ion-exchangers using 80 to 90% acetonitrile in the eluents. The compounds are again separated by their increasing hydrophilicity using a salt gradient, typically potassium perchlorate due to its good solubility in aqueous acetonitrile. The latter technique was also successfully applied to separate small polar proteins, such as histones (Lindner et al., 1997). More recently, HILIC–ESI-MS was employed by several groups to separate and quantify folates in human plasma (Garbis et al., 2001), polar compounds in plants (Tolstikov and Fiehn, 2002), amino acids and polar dipeptides in food (Schlichtherle-Cerny et al., 2003), and glycosylated peptides (Hägglund et al., 2004). These studies included also some amino acids, but to our best knowledge, a separation of all proteinogenic amino acids has not been published yet. The objective of the present study was to analyze the hydroxyproline (Hyp) isomers trans-4-, cis-4-, and trans-3-Hyp commonly found in hydrolysates of collagens, the most abundant proteins in mammalians. The method should allow analysis of all proteinogenic amino acids as well as all three Hyp-isomers using a neutral loss scan that increased the specificity for amino acids and allowed quantification.

Experimental

Chemicals

All twenty proteinogenic L-amino acids, (2S,4R)-4-hydroxypyrrolidine-2-carboxylic acid (trans-4-hydroxy-L-proline, trans-4-Hyp), (2S,3S)-3-hydroxypyrrolidine-2-carboxylic acid (trans-3-hydroxy-L-proline, trans-3-Hyp), (2R,4R)-4-hydroxypyrrolidinecarboxylic acid (cis-4-hydroxy-L-proline, cis-4-Hyp), ammonium acetate, 6 mol/l hydrochloric acid (hydrolysis grade), acid soluble collagen Sigma types III (calf skin), VII (rat tail) and VIII (human placenta) as well as β-casein were purchased from Sigma-Aldrich-Fluka GmbH (Steinheim, Germany) at the highest purity available. Acetonitrile (HPLC grade) was from Fisher Scientific UK Ltd (Leicestershire, UK). Water was purified with an ELGA PURELAB ULTRA system from Vivendi Water Systems Ltd (Bucks, UK).

HILIC–ESI-MS

Amino acids were separated on a TSK-Gel Amide 80 column (15 cm × 2.0 mm, 5 μm; TOSOH Bioscience, Stuttgart, Germany) in HILIC mode using an 1100 series gradient HPLC system (Agilent Technologies GmbH, Waldbronn, Germany) coupled on-line to an API 2000 or API 3000 triple quadrupole mass spectrometer equipped with an ESI source (TurboIonSpray, Applied Biosystems GmbH, Darmstadt, Germany). Eluents were 90% aqueous acetonitrile containing 0.5 mmol/l ammonium acetate (pH 5.5, eluent A) and 60% aqueous acetonitrile containing 2.5 mmol/l ammonium acetate (pH 5.5, eluent B). The amino acids were eluted by a water gradient using two linear increments first from 5 to 60% eluent B for 28.5 min and then to 95% in 5 minutes. The amino acids were detected by a Q1 scan (m/z 70 to 300 amu) or a neutral loss scan (46 amu) in positive ion-mode (6000 V, 350 °C). Collision energy (CE) and collision activated dissociation (CAD) gas flow for the neutral loss scan were set to the instrumental values 20 and 3, respectively.

Gas phase hydrolysis

Approximately 1 mg collagen types III, VII or VIII were dissolved in 1 ml 50 mmol/l aqueous acetate buffer (pH 5.0) or 2 mg β-casein were dissolved in 1 ml water. One hundred-fifty μl of each solution were transferred into a glass vial inlet (SUPELCO, Bellefonte, USA), dried in vacuum and put into a sealable plastic vial containing 150 μl 6 mol/l hydrochloric acid. The plastic vials were tightly closed and the proteins hydrolyzed at 110 °C for 24 h (collagens) or 3 h (β-casein). The hydrolysates were lyophilized and reconstituted with 75 μl eluent A. Ten μl of each solution was injected for the analysis.

Results and discussion

Amino acid standards

A mixture of amino acids was separated in HILIC mode using an amide-column coupled on-line to an ESI-MS. The mass spectrometer was operated in positive ion-mode using a regular mass scan from m/z 70 to 300 to detect the singly charged amino acids. As coeluting amino acids

Table 1. Retention times of a test mixture containing proteinogenic amino and imino acids using a TSK Gel Amide 80 column (150 mm × 2 mm) with a gradient from 5 to 60% eluent B in 28.5 min at a flow rate of 150 µl/min

Amino acid	Retention time
Trp	10.9 min
Phe	11.5 min
Leu	12.4 min
Ile	13.6 min
Met	14.4 min
Tyr	15.4 min
Val	16.4 min
Pro	18.7 min
Ala	21.1 min
Thr	22.1 min
Gly	22.9 min
Glu	23.3 min
Asp	23.9 min
Ser	24.4 min
Gln	24.5 min
Asn	25.0 min

were identified by their mass, it was important to accomplish base line separation for all isobaric amino and imino acids as well as amino acids having similar masses. Such problematic amino acids are Ile, Leu, Asn, and Asp as well as Glu, Gln, and Lys. Furthermore, phenylalanine is isobaric to methionine sulfoxide, which is slowly formed by oxidation of methionine in the presence of air already at room temperature. A linear gradient increasing the water content from 11.5 to 28% within 28.5 min was able to separate most amino acids (Table 1). Only the very hydrophilic amino acids His, Lys and Arg eluted much later at significantly higher water contents up to 40%. Additionally, these alkaline amino acids eluted in broader peaks with significant tailing. Most important, all amino acids were retained on the stationary phase and eluted by the gradient. Cysteine was excluded from this study because of its oxidation and dimerization behavior. All above mentioned amino acids with similar molecular weights were well separated by HILIC (Table 1), whereas coeluting and partially separated amino acids were distinguished by their m/z. All amino acids displayed similar peak intensities. Variations of the injected sample amounts from 50 to 10,000 pmol affected the relative signal intensities only slightly. Due to the low extinction coefficients of the underivatized amino acids UV detection was very insensitive even at 210 nm. At least for the non-aromatic amino acids the detection limit was in the nmol-range. Only post-column derivatization with ninhydrine or OPA (Cunico and Schlabach, 1983) for example could increase the sensitivity to the low pmol-range, as desired for most studies. However, this would demand optimized chromatographic conditions to separate all proteinogenic amino acids, which appear feasible but might be difficult to achieve and was not within the focus of this study.

Instead MS detection was further investigated to analyze complex samples containing also modified amino acids in positive ion-mode. All amino and imino acids included in this study displayed several fragment ions in tandem mass spectrometry (ESI-MS/MS), which mostly were specific for only one or a few of the amino acids. Only the neutral loss of 46 u, which is most likely attributed to the elimination of formic acid, was observed for all studied amino and imino acids at a significant degree, which was already described in the literature for Hyp, Ile, and Leu (Kindt et al., 2003). As this fragmentation typically resulted in the most dominant signal, it was used to selectively detect alpha amino acids (multi-reaction-monitoring, MRM). As other carboxylic acids do not display such a dominant elimination of formic acid, the neutral loss should reduce the background produced by any contaminants present in the reagents or the samples. Furthermore, it is well suited to quantify MS signals. The sensitivity was not negatively affected by this neutral loss scan. In fact, the MS/MS approach should guarantee a similar sensitivity even for contaminated samples, which was for this instrumental setup typically below 50 pmol per amino acid. The instrumental progress in recent years as well as smaller columns could further improve the sensitivity.

As collagens are dominant proteins that can be easily prepared in large quantities, we did not further pursue to increase the sensitivity. More important for the objective of this study was to (i) prove quantification of the method and (ii) obtain a baseline separation of all isobaric compounds including the Hyp isomers. Indeed, trans-4-Hyp, trans-3-Hyp, and cis-4-Hyp as well as the isobaric Leu and Ile were well separated from each other (Fig. 1). The Hyp isomers were even baseline separated allowing their identification based on both the precursor ion mass and the retention time. Having established the separation of all isobaric compounds in the test mixtures with detection limits below 50 pmol, the next aim was quantification. As amino acids are not equally common in proteins, the quantification must be possible for amino acids present at 100 times higher or lower concentrations. Thus, quantification was studied from 100 pmol to 10 nmol for all three Hyp-isomers targeted in this study. In a single dilution series injecting all three Hyp isomers in six steps at concentrations from 100 to 10,000 pmol, the recorded intensities displayed a linear correlation (correlation coefficients 0.991 to 0.997), with a slight saturation tendency

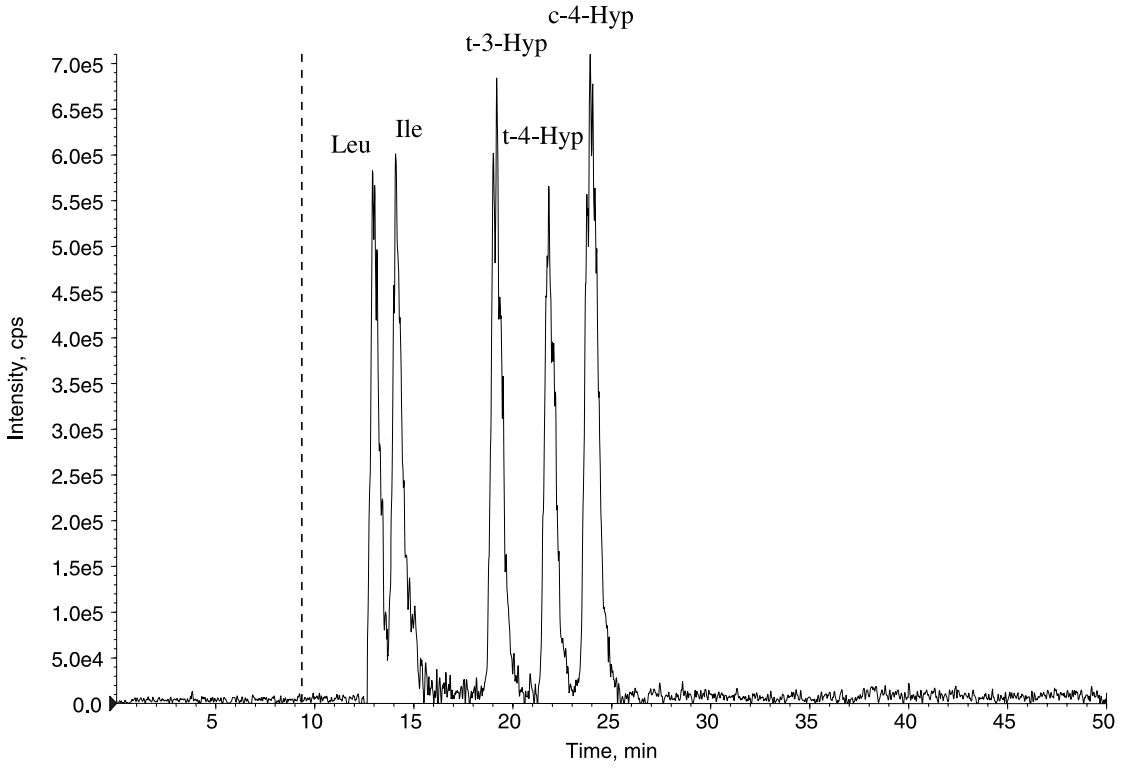

Fig. 1. Extracted ion HILIC–ESI-MS chromatogram of hydroxyproline analyzing a mixture of 19 proteinogenic amino acids (except Cys), both 4-Hyp isomers, and trans-3-Hyp using a neutral loss scan of 46 in positive ion-mode. TSK Gel Amide 80 column (150 mm × 2 mm) with a gradient from 5 to 60% eluent B in 28.5 min at a flow rate of 150 µl/min

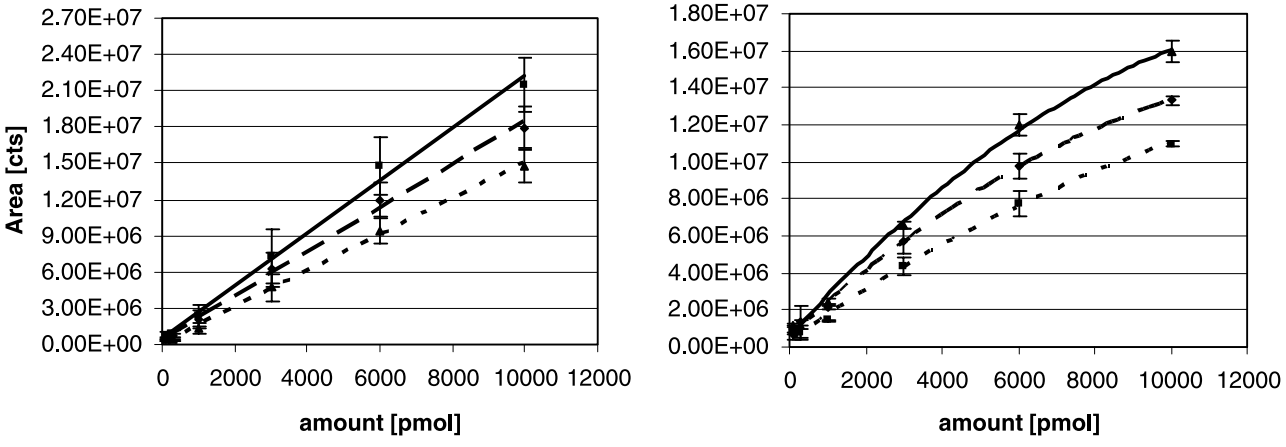

Fig. 2. Signal areas of all three Hyp isomers eluted from a TSK Gel Amide 80 column in HILIC mode depending on the injected sample amounts from 100 pmol to 10 nmol recorded in triplicates using the API3000 operated in positive ion-mode using a neutral loss scan of 46 u. The analytes were eluted by a linear gradient from 5 to 60% eluent B in 28.5 min at a flow rate of 150 µl/min. The left figure shows a linear regression of the obtained data using $2176x + 509692$ (cis-4-Hyp, square, full line), $1807x + 404586$ (trans-3-Hyp, diamond, dashed line), and $1491x + 124851$ (trans-4-Hyp, triangle, dotted line) with regression coefficients $R^2 = 0.994$, 0.996, and 0.998 respectively. The standard deviations among the triplicates were below 20% for each sample amount. The right figure shows a polynomial fit of order two using Asn as internal standard (1000 pmol) using $-0.0758x^2 + 2308x + 556412$ (cis-4-Hyp, triangle, full line), $-0.0621x^2 + 1901x + 540822$ (trans-3-Hyp, diamond, dashed line), and $-0.0368x^2 + 1435x + 353109$ (cis-4-Hyp, square, dotted line) with regression coefficients $R^2 = 0.998$, 0.999, and 0.998 respectively. The standard deviations among the triplicates were below 10% for each sample amount

at higher loading amounts (data not shown). The variation among different dilution series was a little bit higher (Fig. 2), but with standard deviations below 20% within the expected range. To compensate variations of the spray conditions and pipetting errors of the individual dilution series, Asn was added as an internal standard, as it is not

present in hydrolyzed samples and well separated by the gradient. Moreover, it is favorable for Hyp-quantification due to its similar polarity (retention times) and charge (neutral at pH 7). Thereby, the standard deviations were reduced below 10% (Fig. 2), which was similar to the errors obtained for a single dilution series. Whereas a linear fitting was acceptable (regression coefficient better than 0.98), the partial saturation effects at higher concentrations were better described by a square function giving regression coefficients above 0.997 (Fig. 2). Thus absolute quantification is feasible within an error range of 10%. Relative quantification of the Hyp-isomers within a single chromatogram should be even more accurate, as they elute at similar eluent compositions within a short period of time.

Analysis of collagen types

The above described HILIC–ESI-MS approach to quantify amino acids based on a neutral loss scan was tested first on three acid soluble collagen types. From previous studies it is known that proline positions in collagens are mostly hydroxylated in position 4 forming mostly trans-4-Hyp and at a much lower degree in position 3 forming mostly trans-3-Hyp. The latter has been identified at a very low content in all collagen types and is therefore not considered in many studies. Here, an aliquot of the hydrolysate corresponding to typically 20 µg collagen types III, VII or VIII (150 pmol) was analyzed by HILIC–ESI-MS using the standard gradient to determine the ratio of the Hyp isomers in a given collagen type. The ratio of the signal intensities of Ile and Leu was always in good agreement with the expectations based on the collagen sequences indicating the reliable relative quantification of the MS data with an accuracy of 10%. The chromatograms for all three hydrolysates displayed also strong signals for trans-4-Hyp and weak signals for cis-4-Hyp with a relative content of approximately 5%, whereas only collagen VII displayed a weak signal for trans-3-Hyp with a relative content of 3.7% (Fig. 3). The trans-4-Hyp to cis-4-Hyp ratios of all three hydrolysates corresponded very well to earlier investigations that determined about 5% relative amounts for cis-4-Hyp (Erbe and Brückner, 1999).

As the three Hyp-isomers were detected at very different amounts, further problems attributed to the acid hydrolysis have to be considered. For example, Ser and Thr are partially destroyed by the harsh conditions. For neither of the Hyp isomers a significant decomposition during acid hydrolysis was obtained on the amino acid or peptide level within 24 h. As the Hyp isomers are only found in repetitive GPP sequence motifs, they should all be released at very similar rates independent of the hydro-

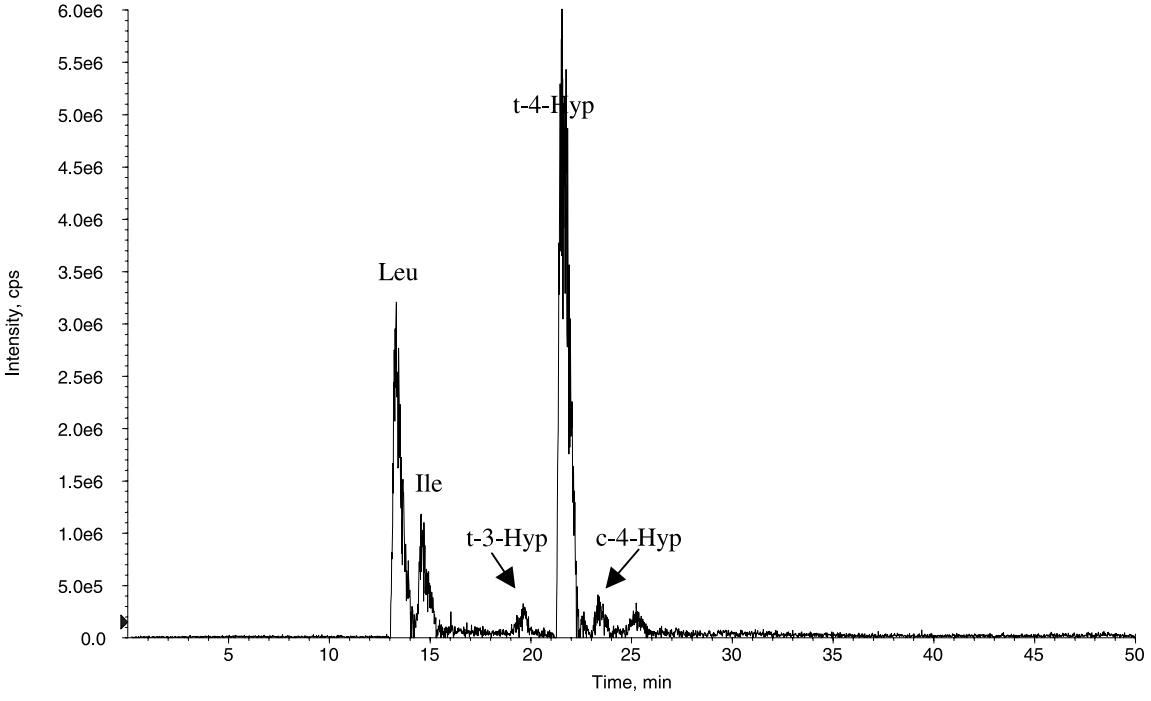

Fig. 3. Extracted ion HILIC–ESI-MS chromatogram of hydroxyproline (m/z 132) analyzing the acid hydrolysate of collagen type VI using a neutral loss scan of 46 u in positive ion-mode. TSK Gel Amide 80 column (150 mm × 2 mm) with a gradient from 5 to 60% eluent B in 28.5 min at a flow rate of 150 µl/min

xylated position and its configuration. Thus, the data obtained for the hydrolysates should closely resemble the in vivo distribution of Hyp isomers in collagen. Only cis-4-Hyp is most likely overestimated, as this signal probably contains both epimers, that is, cis-4-L-Hyp and cis-4-D-Hyp, as Erbe and Brückner (1999) showed that trans-4-L-Hyp partially epimerizes to cis-4-D-Hyp during acid hydrolysis. If formed the two epimers would coelute in HILIC mode, as tested with cis-4-D,L-Hyp (data not shown). However, it should be noted that the total cis-4-Hyp content of the hydrolysate corresponds exactly to the literature, which indicates the potential of the presented HILIC–ESI-MS approach with respect to quantification.

Interestingly, trans-3-Hyp was detected only in the hydrolysate of collagen type VII but not for types III and VIII, even though the peak intensities of the detected trans-4- and cis-4-Hyp isomers were very similar in all three samples. This indicates that collagen type VII contained significantly more trans-3-Hyp than the other two studied collagen types. As the identification and quantification based solely on the neutral loss scan other coeluting contaminants can be ruled out. The same applies to other racemized amino acids, as these coelute with the corresponding L-amino acids. Epimerization of cis-3-L-Hyp yielding trans-3-D-Hyp, which should coelute with trans-3-L-Hyp, appears unlikely, as cis-isomers are present at much lower contents than the corresponding trans-isomers. Furthermore, this side reaction would affect all collagen hydrolysates in the same way, as it depends solely on the hydrolysis time (Erbe and Brückner, 1999). Taken together, collagen type VII had a significantly higher relative trans-3-Hyp content than collagen types III and VI, whereas both 4-Hyp isomers were present in all three collagens at similar contents. This is an interesting observation, as it directs towards specific hydroxylation of some collagen types by 3-hydroxylases.

Analysis of β-casein

The potential of HILIC–ESI-MS to analyze protein hydrolysates without derivatization for posttranslationally modified amino acids was further investigated for phosphoproteins. Thus, β-casein was hydrolyzed with 6 mol/l hydrochloric acid for 3 h in order to identify phosphoserine. Due to its negative charge a significantly reduced sensitivity in positive ion-mode was expected, which is even more pronounced for amino-derivatized phosphoamino acids in RPC–ESI-MS analyses. Phosphoserine was detected at m/z 186 at the upper pmol level using the Q1-scan, which was a tenfold loss of sensitivity com-

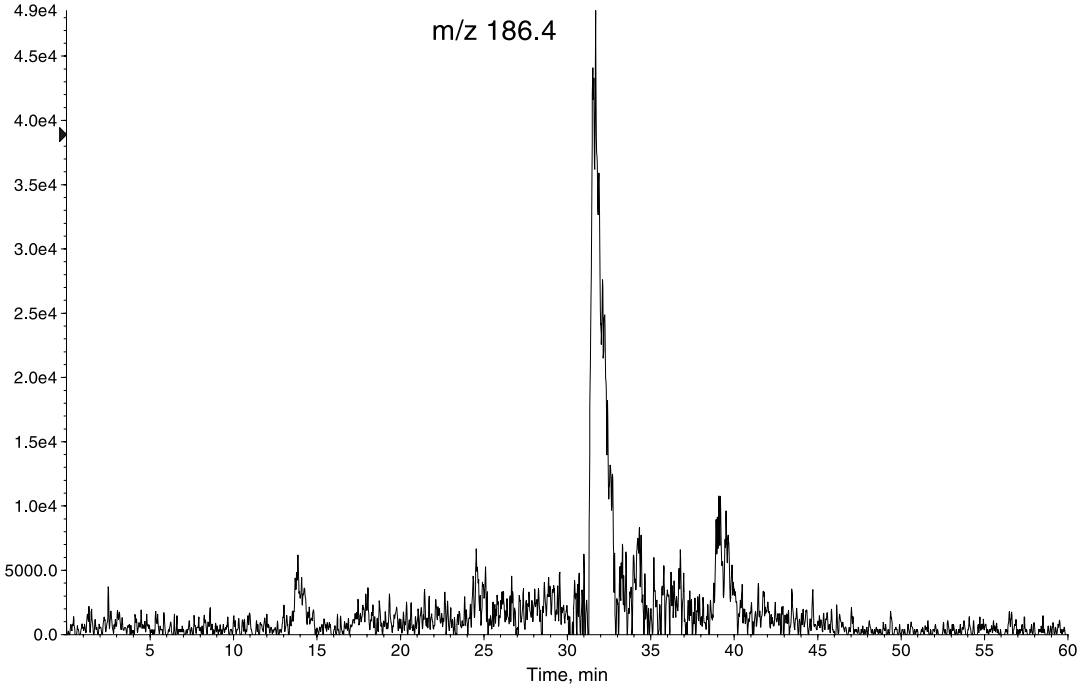

Fig. 4. Extracted ion HILIC–ESI-MS chromatogram of phosphoserine (m/z 186.4) analyzing the acid hydrolysate of β-casein using a neutral loss scan of 98 u in positive ion-mode. TSK Gel Amide 80 column (150 mm × 2 mm) with a gradient from 5 to 60% eluent B in 28.5 min at a flow rate of 150 µl/min

pared to standard amino acids. However, phosphoserine displayed a neutral loss of 98 u corresponding to the elimination of phosphoric acid, instead of 46 u described for all amino acids studied so far. Phosphoserine was identified at a retention time of 31.5 min (Fig. 4) in the casein hydrolysate. Negative ion-mode did not provide a higher sensitivity for the phosphoamino acids and was therefore not further investigated. If phosphorylated amino acids are expected, the mass spectrometer has to be operated in a neutral loss scan of 98 u.

Conclusion

All proteinogenic amino acids except cysteine, which was not included in this study, were successfully analyzed by HILIC–ESI-MS using either an m/z scan or a neutral loss scan that increased the specificity of this method. Importantly, all isobaric amino acids were well separated allowing an unambiguous identification of the amino acids. Quantification with Asn as internal standard was possible between 100 pmol and 10 nmol with an error below 10%. The quantification was confirmed for trans-4-, cis-4-, and trans-3-Hyp in the hydrolysates of three different collagen types. The method has also the promise to identify phosphorylated residues, as shown for phosphoserine in β-casein.

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References

Alpert AJ (1990) Hydrophilic-interaction chromatography for the separation of peptides, nucleic acids and other polar compounds. J Chromatogr 499: 177–196

Brückner H, Keller-Hoehl C (1990) HPLC separation of DL-amino acids derivatized with N2-(5-fluoro-2,4-dinitrophenyl)-L-amino acid amides. Chromatographia 30: 621–629

Cohen SA, Bidlingmeyer BA, Tarvin TL (1986) PITC derivatives in amino acid analysis. Nature 320: 769–770

Cunico RL, Schlabach T (1983) Comparison of ninhydrin and o-phthalaldehyde post-column detection techniques for high-performance liquid chromatography of free amino acids. J Chromatogr A 266: 461–470

Einarsson S, Josefsson B, Moller P, Sanchez SD (1987) Separation of amino acid enantiomers and chiral amines using precolumn derivatization with (+)-1-(9-fluorenyl)ethyl chloroformate and reversed-phase liquid chromatography. Anal Chem 59: 1191–1195

Erbe T, Brückner H (1999) Microwave treatment of dietary gelatin does not generate cis-4-hydroxy-L-proline, an inhibitor of collagen biosynthesis. Z Lebensm Unters Forsch A 208: 424–428

Fenn JB, Mann M, Meng CK, Wong SF, Whitehouse CM (1989) Electrospray ionization for mass spectrometry of large biomolecules. Science 246: 64–71

Garbis SD, Melse-Boonstra A, West CE, van Breemen RB (2001) Determination of folates in human plasma using hydrophilic interaction chromatography-tandem mass spectrometry. Anal Chem 73: 5358–5364

Gusev AI (2000) Interfacing matrix-assisted laser desorption/ionization mass spectrometry with column and planar separations. Fresenius J Anal Chem 366: 691–700

Hägglund P, Bunkenborg J, Elortza F, Jensen ON, Roepstorff P (2004) A new strategy for identification of N-glycosylated proteins and unambiguous assignment of their glycosylation sites using HILIC enrichment and partial deglycosylation. J Prot Res 3: 556–566

Horning EC, Carroll DI, Dzidic I, Haegele KD, Horning MG, Stillwell RN (1974) Liquid chromatograph-mass spectrometer-computer analytical systems. Continuous flow system based on atmospheric pressure ionization mass spectrometry. J Chromatogr 99: 13–21

Kindt E, Gueneva-Boucheva K, Rekhter MD, Humphries J, Hallak H (2003) Determination of hydroxyproline in plasma and tissue using electrospray mass spectrometry. J Pharm Biomed Anal 33: 1081–1092

Lee KS, Drescher DG (1978) Fluorometric amino acid analysis with o-phthaldialdehyde (OPA). Int J Biochem 9: 457–467

Lin JK, Lai CC (1980) High performance liquid chromatographic determination of naturally occurring primary and secondary amines with dabsyl chloride. Anal Chem 52: 630–635

Lindner H, Sarg B, Helliger W (1997) Application of hydrophilic-interaction liquid chromatography to the separation of phosphorylated H1 histones. J Chromatogr A 782: 55–62

Raffaelli A, Saba A (2003) Atmospheric pressure photoionization mass spectrometry. Mass Spectrom Rev 22: 318–331

Robb DB, Covey TR, Bruins AP (2000) Atmospheric pressure photoionization: an ionization method for liquid chromatography-mass spectrometry. Anal Chem 72: 3653–3659

Roturier JM, Le Bars D, Gripon JC (1995) Separation and identification of hydrophilic peptides in dairy products using FMOC derivatization. J Chromatogr 696: 209–217

Schlichtherle-Cerny J, Affolter M, Cerny C (2003) Hydrophilic interaction liquid chromatography coupled to electrospray mass spectrometry of small polar compounds in food analysis. Anal Chem 75: 2349–2354

Schwarz EL, Roberts WL, Pasquali M (2005) Analysis of plasma amino acids by HPLC with photodiode array and fluorescence detection. Clin Chim Acta 354: 83–90

Tolstikov VV, Fiehn O (2002) Analysis of highly polar compounds of plant origin: Combination of hydrophilic interaction chromatography and electrospray ion trap mass spectrometry. Anal Biochem 301: 298–307

Tornkvist A, Nilsson S, Amirkhani A, Nyholm LM, Nyholm L (2004) Interference of the electrospray voltage on chromatographic separations using porous graphitic carbon columns. J Mass Spectrom 39: 216–222

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