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# Proteolysis and autolysis of proteases and the detection of degradation products in doping control

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**The determination of protein- or peptide-based performance-enhancing pharmaceuticals has gained increasing attention in sports drug testing in recent decades. Proteases possess a high potential for use in the manipulation of regular urine specimens, interfering with established doping control procedures for proteins or peptides. Hence, the present approach was developed to identify degradation products directly from proteases with exogenous origin in urine samples by means of SDS-PAGE and capillary liquid chromatography – Orbitrap (tandem) mass spectrometry. The mass spectrometric identification was accomplished by database search considering the accurate monoisotopic precursor mass with data dependent MS/MS analysis and no setting for specific cleavage site(s) or enzyme(s). The main characteristics of the method were shown in an exemplary way for the proteases Papain, Bacillolysin, Trypsin and Subtilisin with sufficient results for specificity, limit of detection, working range, precision and recovery after incubation. All experiments were performed with fortified urine samples under consideration of assumed realistic conditions for adulteration by cheating sportsmen.** 

**Keywords:** sport, doping, mass spectrometry, manipulation, orbitrap

## Introduction

The World Anti-Doping Agency (WADA) prohibits 'chemical and physical manipulation' of urine samples as well as the use of doping agents for performance enhancement.[1] This manipulation includes catheterization as well as urine substitution or alteration and, therefore, also covers the use of proteases. Proteolytic enzymes degrade proteins. The use of proteases for urine alteration complicates or even inhibits the detection of peptide hormones such as erythropoietin, human chorionic gonadotropin or insulin in doping control.[2–6] A variety of proteases are readily available as pharmaceutical products containing one or more such enzymes; in addition, these agents are common components of washing powder. These products usually contain proteases with animal, herbal or fully synthetic origin, which provides the opportunity to detect non-human, xenobiotic proteases in urine. Human endogenous proteases are rare in urine and mass spectrometric detection was achieved only when large amounts (~200 mL) of urine were prepared for analysis. Trypsinogen, Chymotrypsinogen and Collagenase were identified in urine earlier[7,8] but did not seem to have proteolytic activity as intact urinary proteins were detected. The problem of erythropoietin (EPO) detection after manipulation with proteases, was recognized and investigated earlier, and the detection of urine manipulation was performed by trypsin digestion of the intact proteases (for example, Chymotrypsin, Papain and Trypsin) entrapped and separated on SDS-PAGE gels.[9,10]

The aim of this work is to develop a method that enables the detection of degradation products of the proteases in urine by LC-MS(/MS) even if no intact protease is visible on SDS-PAGE. Doping control analysis requires a distinct identification of the misused protease, which means that SDS-PAGE alone and the absence of a normal protein pattern are not adequate to report an adverse analytical finding. Therefore, the SDS-PAGE method described earlier[9] is applied to screen for urine samples without commonly observed protein patterns, and a LC-MS/MS-based assay was developed to confirm the presence of protease degradation products in doping control specimens.

## Materials and Methods

Proteases (Subtilisin A (P5380), Papain (P4762), Trypsin (T4665)), as well as dithiothreitol (DTT) and trifluoroacetic acid (TFA, both analytical grade) were purchased from Sigma (Deisendorf, Germany). Bacillolysin (P1236) from Sigma (Deisendorf, Germany) was obtained as protease solution from *Bacillus amyloliquefaciens* with a lot related activity of 0.90 U/g. Solid-phase extraction cartridges OASIS HLB (60 mg, 3 $cm^3$) were bought from Waters (Eschborn, Germany) and Amicon centrifugal filters (cut-off 10 kDa) were obtained from Millipore (Schwalbach, Germany). Twelve per cent Bis-Tris gels, 3-(*N*-morpholino)propanesulfonic acid (MOPS) running buffer and lithium dodecyl sulphate (LDS) sample buffer were from Invitrogen (Karlsruhe, Germany) and Coomassie Blue gel stain from Pierce (Rockford, IL, USA). Methanol, acetonitrile and acetic acid (all analytical grade) were from Merck (Darmstadt,

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Germany). All aqueous buffers and solutions were prepared in MilliQ water.

### Urine samples

All urine specimens analysed within the validation process were spontaneous urine samples obtained from healthy male and female volunteers and were stored at 4 °C until analysis. Urine samples were heated to 37 °C prior to addition of the respective protease with subsequent cooling to room temperature to simulate authentic conditions for urine adulteration by cheating sportsmen.

### SDS-PAGE of urine samples

Screening for unusual protein patterns in urine samples was accomplished by concentrating 4 mL of urine in a centrifugal filter (10 kDa cut-off, 25 min, 4000 g) to approximately 50 µL. A volume of 20 µL of the retentate was mixed with 7 µL of LDS sample buffer (4×) and 3 µL of DTT and heated for 10 min at 70 °C. After SDS-PAGE (12% Bis-Tris gel, MOPS running buffer, 125 V) gels were stained with Coomassie Blue.

### Liquid chromatography – (tandem) mass spectrometry

Sample preparation was performed by solid phase extraction (SPE) with 1 mL of urine added to an OASIS HLB (60 mg, 3 $cm^3$) SPE cartridge that was preconditioned consecutively with 2 mL of acetonitrile and 2 mL of water. The cartridge was washed with 2 mL of water and elution of analytes was accomplished with a mixture of water/acetonitrile (1.4 mL, 20/80, v : v). The eluate was evaporated to dryness in a vacuum centrifuge prior to reconstitution with 100 µL of acetic acid (2%). Liquid chromatography was performed on an Agilent (Böblingen, Germany) 1100 capillary HPLC equipped with a Zorbax 300 SB trapping column (5 × 0.3 mm, particle size 3.5 µm, 50 µL/ min) and a Zorbax 300 SB-C18 analytical column (50 × 0.3 mm, particle size 3.5 µm, 10 µL/ min, both Agilent). Solvent A was 0.1% acetic acid containing 0.01% of trifluoroacetic acid, and solvent B was composed of 80% acetonitrile with 0.1% of acetic acid and 0.01% of trifluoroacetic acid. The following gradient was used: 0–2 min 95% A, 2–25 min 60% A, 25–34 min 3% A, 34–44 min 3% A, re-equilibration 16 min 95% A. Mass spectrometric analysis was done on a high-resolution/high-accuracy instrument (Thermo LTQ Orbitrap, Bremen, Germany) with electrospray ionization in positive mode and an ionization voltage of 3.5 kV. The resolution was set to 30 000 (FWHM) and the collision energy for automated data-dependent MS/MS experiments, which were performed using the linear ion trap mass analyser, was 35% (arbitrary units, Xcalibur software version 2.0, Thermofisher Scientific, 2006). Calibration of the instrument was performed prior to measurement using the manufacturer's calibration mixture to ensure accurate mass determination.

### Data analysis

Bioworks 3.3 including the Swissprot/Uniprot database (2006, Thermo, Bremen, Germany) was used for the evaluation of LC-MS/MS data. The database search was not restricted to any particular enzyme or species. The identification of proteins was considered successful if at least two peptides were detected and the peptide masses did not deviate more than 10 ppm from theory. Mass tolerance for product ions was set to 0.8 Da.

### Standard solutions

Proteases standard solutions were prepared in an aqueous concentration of 1 mg/mL and stored at −20 °C. They were used at most three times to avoid a loss of activity due to freeze and thaw cycles. The Bacillolysin stock solution was stored at 2–8 °C and used without further dilution as distributed by the provider.

### Validation

The method is supposed to be used as analytical tool to detect misuse of proteases for urine alteration. Therefore a validation for qualitative purposes was performed under consideration of the items specificity, working range, repeatability, robustness, stability and limit of detection.

### Specificity

Ten different blank samples with known absence of exogenous proteases were prepared for LC-MS/MS measurement to prove that no peptides derived from xenobiotic proteases were identified by the software. The specificity of the SDS-PAGE method was shown earlier by preparation of more than 100 blank urine samples.[9]

### Limit of detection (LOD)

The limit of detection for SDS-PAGE was considered to be the lowest amount of protease spiked to blank urine samples that resulted in proteolytic degradation of urinary proteins and therefore the detection of atypical protein patterns after 1D gel electrophoresis or the lowest amount that yields characteristic protease bands on the gel. Protease amounts of 5 µg/mL for Trypsin, 1 µg/mL for Papain and Subtilisin and 0.25 µL/mL for Bacillolysin were added to 4 mL of urine.

The LOD of the LC-MS procedure was determined in order to evaluate the performance of the mass spectrometric identification of the proteases. Therefore, blank urine samples were fortified with the respective amount of protease (10 µg/mL for Trypsin, 5 µg/mL for Papain, 20 µg/mL for Subtilisin, 1 µL/mL for Bacillolysin) and analysed with six repetitions.

The limit of detection was defined as the amount of protease that is required to detect at least two protease-specific peptides from autolysis with accurate monoisotopic precursor mass and respective product ion mass spectra.[11]

### Working range

In order to define a working range of the LC-MS/MS method, aliquots of blank urine samples were fortified with different amounts of proteases. This approach was performed for Bacillolysin by adding 0.5, 1, 2, 5 and 10 µL, for Papain by adding 1, 5, 10, 20, 50 and 100 µg and for Subtilisin by adding 5, 10, 20 and 50 µg of protease to 1 mL of urine, each.

### Repeatability and robustness

To evaluate the repeatability of the LC-MS/MS measurement, six samples were fortified with defined amounts of proteases (100 µg/mL Papain, 2 µL/mL Bacillolysin) and the identified peptides were compared. The influence of the presence of two proteases in one sample was shown by adding 0.1, 0.5, 1, 2 and 5 µg/mL of Subtilisin to urine specimens that additionally contained 2 µl/mL of Bacillolysin, which is produced in the same bacteria. This approach provides data for robustness of the method when protease cocktails are used to alter the doping control sample.

### Recovery-period after incubation of urine samples

For the evaluation of the stability of the selected proteases in urine, aliquots of blank urine specimens were fortified with Papain (10 µg/mL) or Bacillolysin (1 µL/mL) and analysed after incubation at room temperature for 0, 4, 8 and 24 hours with two repetitions.

### Stability of blank urine samples

To probe for the alteration of urine samples at different pH and different storage temperatures, two different urine specimens were stored at room temperature, 4 °C and −20 °C for 0, 1, 4 and 7 days after adjustment to pH 5, 6, 7 or 8. This was tested to prove the stability of the urine sample itself and avoid suspicious samples on SDS-PAGE due to degradation of urinary proteins from low-concentration endogenous proteases that may be excreted into urine.

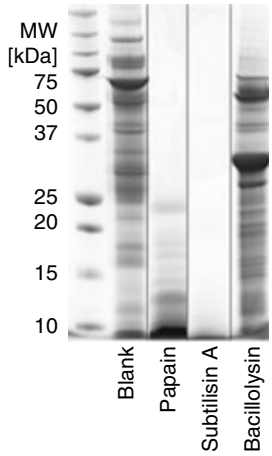


**Figure 1.** SDS-PAGE of a blank urine and the same urine fortified with different proteases (Papain 2 µg/mL, Subtilisin 1 µg/mL, Bacillolysin 1 µL/mL, incubation 1h 37 °C).

## Results and Discussion

Manipulation of urinary specimens in sports drug testing with proteolytic enzymes is a simple and highly effective way to disable common peptide and protein analysis. Thus, an analytical approach for the direct identification of exogenous proteases in urine provides a helpful advance in doping control. The present study was initiated to fill a gap in existing test procedures by providing an unambiguous identification of proteases with artificial origins in urine. An approach based solely on LC-MS/MS was investigated to enable the detection of proteases that are not visible as characteristic bands in SDS-PAGE, as described and used for doping control purposes earlier.[9]

Figure 1 shows a SDS-PAGE gel with a blank urine, with and without the addition of different proteases. It gives an overview of the protein pattern after treatment with different proteases. Enrichment of a urine sample with Bacillolysin results in a characteristic protein band at about 30 kDa, but Subtilisin treatment results in fast proteolysis and autolysis leaving hardly any protein band on the gel. For the detection and identification of proteases such as Subtilisin, an upgrade of the SDS-PAGE method was required. The present approach allows the detection of any of the studied proteases by LC-MS/MS in urine samples using proteolysis or autolysis products that are recovered from urine by means of solid phase extraction. The analysis of peptides and proteins with mass spectrometry-based methods is a commonly used technique with well established criteria for identification of respective target analytes.[11] Unfortunately, the determination of proteases in urine represents an exceptionally complex task due to their sustained biological activity in the matrix. The nature of simultaneous proteolytic and autolytic processes and mixtures of proteases are characterized by non-specific cleavage sites and, thus, the occurrence of degradation products is inhomogeneous and influenced by various parameters (such as temperature, time and pH).[12] These facts require modified, but still highly specific, criteria to identify the target analytes based on their degradation products occurring in urine samples. In contrast to typical bottom-up approaches where hydrolysis of proteins is accomplished with endoproteinases (such as Trypsin, Lys-C and Glu-C) that cleave at specific amino acid sites, in the present study all possible cleavages need to be taken into account in order to identify characteristic peptides. Accurate determination of the monoisotopic precursors with data-depended MS/MS analysis under consideration of the charge state of the respective precursor ion enabled the detection and identification of peptides with unknown cleavage sites. Acquired data were subsequently subjected to a database search without selection of particular enzymes (or cleavage sites) because manual evaluation of the data is hindered by the enormous volume of information.

Figure 2 demonstrates main parts of the database output from a urine sample fortified with 1 µL/mL of Bacillolysin. Five peptides were identified with a mass error $< 10$ ppm for the respective doubly charged precursor ion and the total sequence coverage was 25%. The amino acid sequences highlight the identified peptides, which are distributed across the entire protease.

Fig. 3 gives an example for the raw data that the database utilizes for the evaluation and identification of the respective peptides. Figure 3a shows the extracted ion chromatogram for the monoisotopic precursor ion (range *m/z* 883.92–884.01) and the FTMS spectrum of the precursor ion at *m/z* 883.97, which indicates the charge state and the error (DeltaM) of <5 ppm. The identified

**Table 1.** Validation results for the LC/MS analysis

| | MW (Da) | Amino acids | LOD (µg/ml) | Working range (µg/ml) | Precision $n = 6$ | Recovery-period $n = 2 + 2 + 2 + 2$ |
|---|---|---|---|---|---|---|
| Bacillolysin | 32710,9 | 300 | 1* | 1 to 10* | 26,7% | 24 h |
| Papain | 23413,6 | 312 | 5 | 5 to 100 | 4,3% | 4 h |
| Subtilisin | 27284,6 | 274 | 20 | 20 to 50 | n.a. | n.a. |
| Trypsin | 23290,3 | 220 | 10 | 10 to 50 | n.a. | n.a. |

* (µL/mL)

Bacillolysin precursor (EC 3.4.24.28) (Neutral protease)

| Peptide | MH+ | DeltaM (ppm) | z | Coverage (%) | MS/MS-Ions (found/possible)* |
|---|---|---|---|---|---|
| IQSARDLYGSQDAASVE | 1809.86 | 6.02 | 2 | 5.64 | 21/32 |
| LSKPTGTQIITYD | 1436.76 | 1.51 | 2 | 4.33 | 17/24 |
| VSLNISSESGKYVLRD | 1766.93 | 1.71 | 2 | 5.36 | 20/30 |
| LSKPTGTQIITYDL | 1549.85 | 4.11 | 2 | 4.65 | 18/26 |
| VYLTPSSTFKDAKAAL | 1711.93 | 2.31 | 2 | 5.31 | 19/30 |
| | | | Sum | 25.3 | |

AATTGTGTT LKGKT **VSLN** **SSESGKYVL** **DLSKPTGTQ**
**ITYD** QNREY NLPGTLVSST TNQFTTSSQR AAVDAHYNLG
KVYDYFYQKF NRNSYDNKGG KIVSSVHYGS RYNNAAWIGD
QMIYGDGDGS FFSPLSGSMD VTAHEMTHGV TQETANLNYE
NQPGALNESF SDVFGYFNDT EDWDIGEDIT VSQPALRSLS
NPTKYGGQPDN FKNYKNLPNT DAGDYGGVHT NSGIPNKAAY
NTITKIGVNK AEQIYYRALT **VYLTPSSTF** **DAKAALIQS**
**RDLYGSQDAA** **SVE** AAWNAVG L

**Figure 2.** Database search results of a urine sample fortified with 1 µL of Bacillolysin solution. Below: Amino acid sequence of Bacillolysin. Identified peptides are printed in bold. * Sum of b- and y-ions identified in the MS/MS spectra by the software.

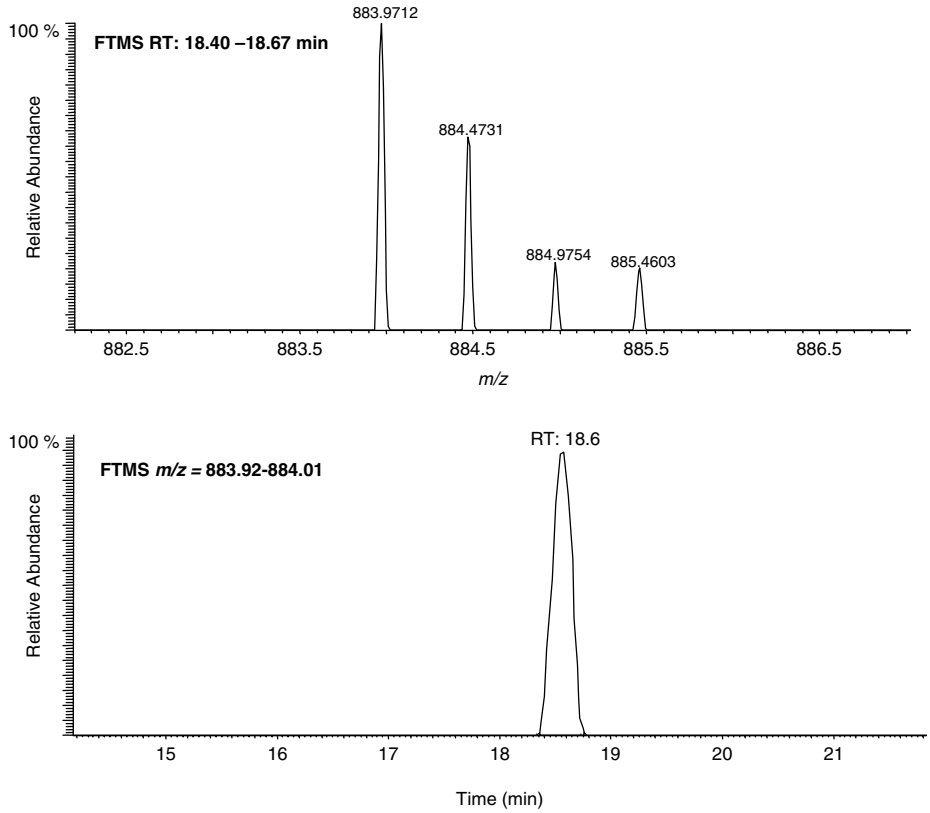


**Figure 3a.** FTMS Orbitrap full scan mass spectrum and extracted ion chromatogram (below) of the monoisotopic precursor ion of the peptide VSLNISSESGKYVLRD, analysed in a urine sample fortified with 1 µL of Bacillolysin solution.

peptide is related to the amino acid sequence of Bacillolysin and was also found in the database search as illustrated in Fig. 2. Finally, Fig. 3b demonstrates the corresponding tandem mass spectrum and the detected y- and b-ions as assigned by the software.

## Validation

The main results of the validation are summarized in Table 1. The validation of the method is described for Trypsin, Papain, Subtilisin and Bacillolysin. Trypsin is the most popular protease in protein analysis and is readily available. Papain and especially Subtilisin are proteases that are often not identified by trypsin digestion after SDS-PAGE because of very fast autolysis rates. They therefore have greater potential to be misused and their detection would not be possible with the SDS-PAGE method published earlier.[9] Bacillolysin is produced in the same bacteria as Subtilisin and may be detected as an artefact of the use of Subtilisin when badly purified Subtilisin is used.

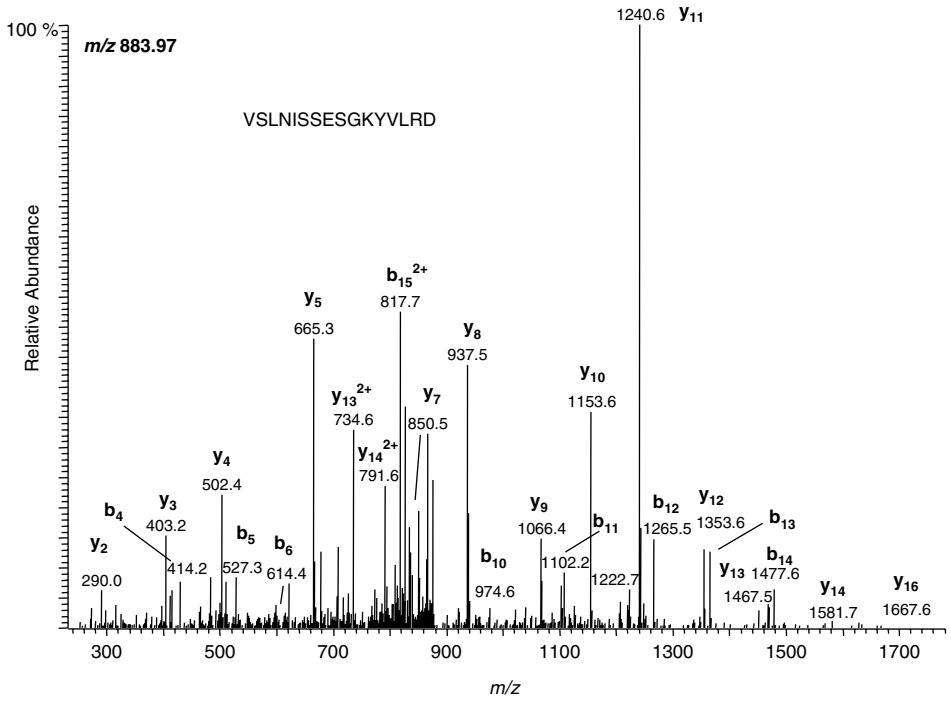


**Figure 3b.** Data-dependent MS/MS spectrum of the doubly charged precursor ion at *m/z* 883.97.

### Specificity

The database search results of urine samples, which were collected from healthy volunteers and measured for specificity, did not yield any protease, and no proteolytic activity was visible on SDS-PAGE. Electrophoretic analysis yielded common distributions and patterns of endogenous proteins. In LC/MS measurements, only human Uromodulin was identified with at least two peptides. Neither the specificity measurements of blank urine samples nor samples fortified with different proteases for the validation measurements yielded non-human proteins in the database search that did not correspond with the detected proteases.

### Limit of detection

The limit of detection for LC-MS/MS analyses was determined for the proteases Papain, Subtilisin and Bacillolysin by determination of the monoisotopic precursor ions and corresponding MS/MS spectra of at least two peptides deriving from the amino acid sequence of the protease. Despite the strong influence of storage conditions, the present approach led to LODs of 5 µg/mL for Papain, 10 µg/mL for Trypsin, 1 µL/mL for Bacillolysin and 20 µg/mL for Subtilisin. The LODs for LC-MS/MS analyses were also shown to be valid for SDS-PAGE analyses (with even lower LODs) with characteristic bands or disappearance of endogenous proteins on Coomassie-stained gels. Samples fortified with 5 µg/mL of Trypsin, 1 µg/mL of Papain, 1 µg/mL of Subtilisin or 0.25 µL/mL of Bacillolysin provided significantly altered protein patterns on the gel.

### Working range

The working range should define the approximate concentration of protease in urine that will produce reliable results and prevent carry-over effects in liquid chromatography. Besides the analytical aspects, these concentrations are also attributable to the expected amounts in adulterated urine specimens. The working range was found to be 5 to 100 µg/mL for Papain, between 1 and 10 µL/mL for Bacillolysin, 10 to 50 µg/mL for Trypsin and 20 to 50 µg/mL for Subtilisin. These concentration ranges yielded reliable results considering the identification of a sufficient number of peptides. Especially for Subtilisin, the working range and LOD are influenced by various external conditions (such as hydrolysis time and temperature) and, thus, a concentration of 20 µg/mL is required for an unambiguous identification with LC-MS/MS.

### Repeatability

Despite the complex biological processes and their dynamic nature, the repeated preparation of fortified samples of Papain (100 µg/mL) and Bacillolysin (2 µL/mL) yielded a sequence coverage of 32% to 36% and 35% to 69%, respectively.

### Recovery period after incubation

Bacillolysin added to a blank urine sample was still detectable after 24 hours at room temperature and Papain was stable for at least 4 hours, which means that incubation times or times during sample preparation do not interfere with the analysis due to autolysis in the indicated range. Generally, the best storage conditions were −18 °C or less in order to prevent autolysis of the proteases in urine. This is also valid for Trypsin, Subtilisin and other proteases that were not tested for recovery within this study.

### Stability of blank urine samples

Proteins in blank urine samples that were stored at +4 °C or −20 °C did not show degradation after seven days of storage at any tested pH (5, 6, 7, 8). In contrast, samples stored at room temperature showed clear protein degradation after four days on SDS gels at

pH 7 and 8. Samples adjusted to pH 5 or 6 prior to incubation were degraded slower and showed only slight changes after one week of storage.

### Robustness

Interesting effects were detected when mixtures of proteases, especially Subtilisin and Bacillolysin, which are produced in the same bacteria representing an alkaline and a neutral protease of *Bacillus sp.*, were used in combination.[13] The presence of Bacillolysin and Subtilisin in the same sample obviously improved the LOD for Subtilisin. Combined addition of 2 µL/mL of Bacillolysin and 2 µg/mL of Subtilisin in one urine sample yielded a sequence coverage of 11% for Subtilisin, while the limit of detection for Subtilisin alone was determined at 20 µg/mL. Due to the presence of Bacillolysin as an impurity in *Bacillus sp.* extracts, this could help to detect proteases in urine samples by either lowering the LOD for Subtilisin or by detection of Bacillolysin.

## Conclusions

Proteases are substances that can be used to manipulate urine samples in doping control testing. Their addition to urine impedes the detection of peptides and proteins in urine that may have been used for illegal performance enhancement. The method developed here improves the detection method described earlier by enabling the detection of protease degradation products from urine samples when no intact protease is visible on SDS-PAGE. This allows the detection of proteases such as Subtilisin, which autolyse very quickly, and also facilitates detection when protease cocktails are used. The more experience is gained with exogenous proteases in urine samples the longer the list of detected proteases will become and the method easily allows the detection of new or other proteases or mixtures. Despite the fact that identification focused on selected proteases in this project, the method's principle is not limited to those and transferring it to other target proteases with exogenous origin is feasible. Furthermore, samples that should be tested for proteases could be selected by choosing samples from EPO analysis, where no EPO is detected in the doping control analysis. They could then be applied to SDS-PAGE and, if suspicious, to LC-MS/MS analysis.

### Acknowledgements

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## References

[1] World Anti-Doping Agency, The 2008 Prohibited List. Available at www.wada-ama.org/rtecontent/document/2008_List_En.pdf, accessed 26 January **2009**.
[2] F. Lasne, L. Martin, N. Crepin, J. de Ceaurriz. *Anal. Biochem.* **2002**, *311*, 119.
[3] M. Kohler, C. Ayotte, P. Desharnais, U. Flenker, S. Lüdke, M. Thevis, E. Völker-Schänzer, W. Schänzer. *Int. J. Sports Med.* **2008**, *29*, 1.
[4] A. Thomas, M. Thevis, P. Delahaut, A. Bosseloir, W. Schänzer. *Anal. Chem.* **2007**, *79*, 2518.
[5] M. Thevis, A. Thomas, P. Delahaut, A. Bosseloir, W. Schänzer. *Anal. Chem.* **2006**, *78*, 1897.
[6] M. Bidlingmaier, Z. Wu, C. J. Strasburger. *Baillieres Best Pract. Res. Clin. Endocrinol. Metab.* **2000**, *14*, 99.
[7] J. Oh, J. H. Pyo, E. H. Jo, S. I. Hwang, S. C. Kang, J. H. Jung, E. K. Park, S. Y. Kim, J. Y. Choi, J. Lim. *Proteomics.* **2004**, *4*, 3485.
[8] C. S. Spahr, M. T. Davis, M. D. McGinley, J. H. Robinson, E. J. Bures, J. Beierle, J. Mort, P. L. Courchesne, K. Chen, R. C. Wahl, W. Yu, R. Luethy, S. D. Patterson. *Proteomics* **2001**, *1*, 93.
[9] M. Thevis, J. Maurer, M. Kohler, H. Geyer, W. Schänzer. *Int. J. Sports. Med.* **2007**, *28*, 545.
[10] S. Lamon, N. Robinson, P. E. Sottas, H. Henry, M. Kamber, P. Mangin, M. Saugy. *Clin. Chim. Acta.* **2007**, *385*, 61.
[11] M. Thevis, J. A. Loo, R. R. Loo, W. Schänzer. *Rapid Commun. Mass Spectrom.* **2007**, *21*, 297.
[12] H. Schlüter, D. Hildebrand, C. Gallin, A. Schulz, J. Thiemann, M. Trusch. *Anal. Bioanal. Chem.* **2008**, *392*, 783.
[13] S. J. Cho, S. H. Oh, R. D. Pridmore, M. A. Juillerat, C. H. Lee. *J. Agric. Food Chem.* **2003**, *51*, 7664.