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Evaluation of online carbon isotope dilution mass spectrometry for the purity assessment of synthetic peptide standards

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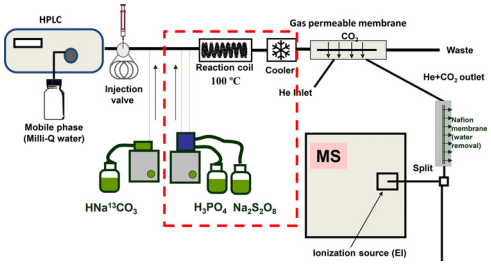
HIGHLIGHTS

- Purity assessment of peptide standards applicable to any water soluble peptide.
- Online ^{13}C isotope dilution mass spectrometry.
- Mass flow chromatogram from measured 44/45 isotope ratios.
- Validation by the analysis of NIST 8327.

GRAPHICAL ABSTRACT



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ABSTRACT

We present a novel method for the purity assessment of peptide standards which is applicable to any water soluble peptide. The method is based on the online ^{13}C isotope dilution approach in which the peptide is separated from its related impurities by liquid chromatography (LC) and the eluent is mixed post-column with a continuous flow of ^{13}C-enriched sodium bicarbonate. An online oxidation step using sodium persulfate in acidic media at 99 °C provides quantitative oxidation to $^{12}CO_2$ and $^{13}CO_2$ respectively which is extracted to a gaseous phase with the help of a gas permeable membrane. The measurement of the isotope ratio 44/45 in the mass spectrometer allows the construction of the mass flow chromatogram. As the only species that is finally measured in the mass spectrometer is CO_2, the peptide content in the standard can be quantified, on the base of its carbon content, using a generic primary standard such as potassium hydrogen phthalate. The approach was validated by the analysis of a reference material (NIST 8327), and applied to the quantification of two commercial synthetic peptide standards. In that case, the results obtained were compared with those obtained using alternative methods, such as amino acid analysis and ICP-MS. The results obtained proved the value of the method for the fast, accurate and precise mass purity assignment of synthetic peptide standards.

1. Introduction

The field of MS-based quantitative proteomics has grown enormously in the last years [1]. Protein quantitative analyses, performed in fields such as clinical chemistry or pharmacology, are mainly based on the hydrolisis of the proteins by enzymatic digestion (e.g., trypsin) and the determination of "proteotypic" peptides by isotope dilution LC–MS/MS. It is clear that for the development of accurate and reliable methods for protein quantification, there is a real need for SI-traceable peptide standards. Unfortunately, the production of high purity peptide standards, with known degrees of hydration and absence of other impurities such as salts, is not easy and in most cases not possible. As a result, peptide standards need to be certified for purity.

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Traditionally, peptide purity is established by LC with UV detection but the difference between the area percent purity and the mass fraction (w/w %) for a peptide standard can be as large as 20–30% [2]. Therefore, the simply gravimetric preparation of a synthetic peptide standard solution is not reliable when a good accuracy is sought [3]. In such cases, the mass fraction of the peptide is usually determined through amino acid analysis (AAA) and the area percent purity obtained by alternative techniques such as CE/LC-UV or MALDI [4]. In the search for better accuracy and precision, the latest AAA procedures involve chemical hydrolysis of the peptides in 6 M HCl followed by quantification of the released amino acids by LC or GC in combination with IDMS using labeled analogs. The traceability of this method is achieved through amino acid standard reference materials that are commercially available [5]. This method has allowed the traceable quantification of various peptide and protein standards with combined expanded uncertainties between 2 and 6%, being the uncertainty associated to the purity of the amino acid standards used the main contribution [3–8]. Although under carefully controlled conditions this procedure provides very good results in terms of accuracy and precision, it is a long, expensive and a labor intensive methodology. It is important to stress that the purity assessment carried out by chromatographic (CE, LC) or MS (MALDI) methods, assumes identical instrumental response for the peptide of interest and its related impurities, which brings more uncertainty to the final quantitation. It seems clear that the development of direct and faster methods for peptide purity assessment is required.

In this regard some attempts have been made using elemental ion sources, specifically the inductively-coupled plasma source with mass spectrometry (ICP-MS). This ion source represents an alternative platform for the quantitative analysis of peptides and proteins as its signal is proportional to the amount of the heteroatom measured, independently of the chemical form in which it is presented, and thus, allowing the use of a single generic standard to calibrate the instrumental response [9]. The main disadvantage of the technique lies on the necessity of an ICP-detectable element (other than C, N, H, O) in the structure of the molecule to be measured. In the best case, peptides with cysteine of methionine can be directly measured following the S signal [10,11]. Alternatively, phosphopeptides [12] or element-labeled peptides using chemical reactions [13,14] can also be used. In this line, the determination of bradykinin by europium labeling and post-column IDMS has been recently reported [15]. On the other hand, LC coupled with chemiluminescent nitrogen detection (CLND), has been also evaluated for peptide content determination of crude synthetic peptides [16]. The response of this detection system is directly proportional to the amount of nitrogen and theoretically, can provide quantitative information without the use of individual analytical standards. Nonetheless, the use of this detector is not widespread, possibly due to its instrumental complexity and lack of robustness [17].

Recently, we described a quantitative detection system for water soluble organic compounds based on post-column isotope dilution, using ^{13}C enriched sodium bicarbonate, followed by online chemical oxidation [18]. Providing that quantitative oxidation is achieved, the only species finally measured is CO_2, and thus, the mass purity of a compound can be computed just by comparison with a generic standard, as the analytical response for both, the analyte and the internal standard will be proportional to the number of carbon atoms present in each molecule. The approach was validated with different low-MW organic standards (sugars, amino acids, drugs) and its compound independent response was demonstrated. The method can be traceable if the quantitation is referred to a certified primary organic standard (i.e., a certified standard of potassium hydrogen phthalate, KHP). Herein, we evaluate this new concept on the purity assessment of peptide standards. Two different approaches are described depending if the peptide can be eluted under 100% aqueous conditions or not. In this latter case a previous purification step by semi-preparative scale LC is proposed. The results were validated using reference materials and by comparison with alternative methods.

2. Materials and methods

2.1. Reagents and materials

Solid enriched $NaHCO_3$ (99% ^{13}C enrichment) was obtained as a high purity chemical reagent (purity >98%) from Cambridge Isotope Laboratories (Andover, Massachusetts, USA). Oxidation reagents, high purity sodium persulfate and solid phosphoric acid, as well as sodium mono and di-hydrogen phosphate (all of them >99%), were purchased from Sigma–Aldrich (St. Gallen, Switzerland). Potassium hydrogen phthalate, KHP (99.98% purity), used as internal standard for quantification, was obtained from Sigma–Aldrich. Ultrapure water (18.2 MΩ cm) was obtained with a Milli-Q system (Millipore, Bedford, MA).

BAX inhibiting peptide v5 standard (VPMLK) was purchased from Sigma–Aldrich (UV–vis purity >97%). Cystatin C T4 tryptic peptide standard (ALDFAVGEYNK) was synthesized by Peptide Synthetics (Funtley, UK) (UV–vis purity >80%), and further purified in our lab by semi-preparative LC. For validation, peptide A from NIST reference material 8327 (DAEPDILELATGYR) with a certified peptide content of 69 ± 11%, was used. All three peptides were obtained as lyophilized powder.

2.2. LC–oxidation–IDMS system

A Thermo Scientific (Bremen, Germany) LC-IRMS instrument consisting of an Accella 600 LC pump, a LC-Isolink interface and a Delta V Advantage mass spectrometer was used. A HTC PAL autosampler (CTC Analytics, Zwingen, Switzerland) was coupled for sample introduction. The LC-Isolink interface consists basically of two independent pumps for the oxidation reagents, a heated reactor, where the chemical oxidation takes place, and a gas permeable membrane, that separates the CO_2 that results from the complete oxidation of the organic compounds, from the liquid phase. The oxidation reagents pumps in the Isolink interface were set at 50 μL min^{-1} each with concentrations of 0.35 M and 1.5 M for the sodium persulfate and the phosphoric acid, respectively, and the reactor was set at a temperature of 99.9 °C. The post-column spike flow was introduced prior the interface by means of a P-500 high precision pump (GE Healthcare, Chalfont St. Giles, U.K.) through a PEEK T piece. All the reagents and mobile phases were degassed under vacuum in an ultrasonic bath, to minimize the background due to dissolved atmospheric CO_2.

2.3. Procedures

2.3.1. Quantification using ^{13}C post-column isotope dilution with internal standard

To quantify the analyte of interest, the isotope ratio $^{12}C/^{13}C$ is continuously monitored as the signal ratio at m/z 44/45 corresponding to the natural abundance $^{12}CO_2$ flow that elutes from the column (after chemical oxidation of the corresponding analyte) and the post-column spike flow ($^{13}CO_2$), respectively. Additionally, a standard of known concentration (KHP) is added to the samples (internal) or injected post-column (external), to calibrate de instrumental response. In addition, the internal standard allows compensating for variations in the volume injected.