QCAL—a Novel Standard for Assessing Instrument Conditions for Proteome Analysis

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If proteome datasets are to be collated, shared, and merged for higher level proteome analyses, there is a need for generally accepted strategies and reagents for optimization and standardization of instrument performance. At present, there is no single protein or peptide standard set that is capable of assessing instrument performance for peptide separation and analysis in this manner. To create such a standard, we have used the recently described QconCAT methodology to generate an artificial protein, QCAL. This protein, a concatenation of tryptic peptides that is expressed in *E. coli*, provides a stoichiometrically controlled mixture of peptides that are amenable to analysis by all commonly used instrumentation platforms for proteomics. (J Am Soc Mass Spectrom 2008, 19, 1275–1280) © 2008 American Society for Mass Spectrometry

The preferred method of peptide characterization is reversed-phase chromatography combined with mass spectrometric analysis. The wide range of chromatographic platforms, mass spectrometers, and data analysis packages make comparison between different instruments challenging. Yet, as we move towards common data standards for publication and archiving of proteomics data, there is an increasing need to normalize such datasets with a common analytical control to permit their meaningful comparison and aggregation. Currently, proteomics researchers use diverse criteria for instrument calibration and optimization. Indeed, many laboratories using multiple mass spectrometers use different standards, often defined by the manufacturer, for calibration and optimization of their individual instruments. In addition, the experimental conditions (solvents, solid-phase, and elution gradient) used for pre-analytical peptide separation are seldom consistent. This makes both intra- and inter-laboratory comparisons of proteomics data almost impossible to perform with any degree of consistency, as is often apparent with the analyses performed by multiple facilities through the Association of Biomolecular Resource Facility (ABRF) (http://www.abrf.org/index.cfm/group.show/Proteomics.34.htm) studies. Increasingly, there is also concern about specifically defining how proteomics datasets are generated [1], and this will include the analytical capability of the instrument used.

Normalization of these systems could be achieved with a universal standard, chosen to assess all aspects of performance and provide a reference analysis that can be fully recorded. Moreover, as experience grows with such a standard, it would be possible to use the behavior of such material as a quality control driver, setting minimal standards for chromatographic resolution, ion selection, fragmentation, and sensitivity. No single protein has yet been characterized that fulfils these requirements. We have recently demonstrated that designer proteins can be used to create a concatenation of tryptic peptides that are surrogate internal standards for absolute protein quantification (QconCAT), [2–4]. However, there are many other feasible applications of designer proteins that can permit exploration of peptide behavior in proteomics and mass spectrometry. Additionally, the low cost and ease of production of the designer protein product makes this preferable to individually synthesizing peptides. Here, we describe the design and analysis of an artificial polypeptide (QCAL1) to optimize and define instrument conditions for peptide analysis by mass spectrometry, whether preceded or not by reversed-phase chromatography.

Experimental

QCAL Construction

The peptide sequences of interest were concatenated in silico and used to direct the design of a gene, codon-optimized for expression in *E. coli*. Additional sequences were added to provide an initiator methionine residue (MGALR) and a His_6 sequence (ALVALVHHHHHH) for affinity purification using Ni-NTA resin. The gene was

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synthesized and cloned into the expression vector pET21a by PolyQuant GmbH (http://www.polyquant.com/).

QCAL Expression and Sample Preparation

QCAL was expressed in *E. coli* BL21 (DE3) cells and purified on a Ni-NTA column as previously described [3], diluted to 1 mg/mL in 50 mM NH_4HCO_3 and digested with 2% (wt/wt) trypsin, overnight at 37 °C. Digested QCAL (1 nmol) was dried by vacuum centrifugation and, for some analyses, guanidination of lysine residues was performed by addition of NH_4OH (7 M, 10 μL) and O-methylisourea (0.5 M in water, 5 μL). After overnight incubation, samples were desalted using C_{18} ZipTips (Millipore, Watford, UK) before MALDI-TOF analysis.

Nanoflow LC-MS Analysis

QCAL was reduced with DTT (10 mM, 56 °C for 1 h) and alkylated (55 mM iodoacetamide, room temperature, dark, 45 min) and the protein precipitated with trichloroacetic acid before reconstitution in 50 mM NH_4HCO_3 and digestion. Tryptic peptides (500 fmol) were desalted in-line using a 5 mm × 300 μm C18 precolumn, before separation by reversed-phase chromatography with a PepMap C18 column (3 μm, 0.075 × 150 mm, 100 Å), both from LC Packings Dionex (Surrey, UK). Chromatography was performed at 200 nL/min using an EASY-nLC (Proxeon, Odense, Denmark) nanoflow system arranged in-line with a QTOF micro (Waters, Milford, MA). The column was equilibrated in 0.2% formic acid (Solvent A) and was developed with 90% acetonitrile/0.2% formic acid (Solvent B); 0% to 20% over 30 min, 20% to 60% over 10 min, and 60% to 100% over 5 min.

Matrix-Assisted Laser-Desorption Ionization-Time of Flight (MALDI-TOF) Mass Spectrometry

MALDI-TOF MS analysis was performed using either the Voyager-DE STR (Applied Biosystems, Foster City, CA) or the Ultraflex II TOF/TOF (Bruker Daltonics, Bremen, Germany) with digested QCAL crystallized with a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% (vol/vol) acetonitrile, 0.1% (vol/vol) trifluoroacetic acid. Detection was performed in reflector mode with delayed extraction.

Fourier Transform Ion Cyclotron Resonance (FT ICR) Mass Spectrometry

Digested QCAL was desalted using a C_{18} peptide trap (Michrom Bioresources, Auburn, CA), dried by vacuum centrifugation and resuspended in 50% (vol/vol) acetonitrile, 0.1% (vol/vol) formic acid to 1 pmol/μL. Peptides were infused and analyzed using a Bruker Daltonics Apex III 9.4T FT ICR mass spectrometer (Billerica, MA) and an electrospray source. Data acquisition was performed with the Bruker Xmass software, version 6.01 (Bruker Daltonics, Bremen, Germany). Mass spectra were collected using 512K data points per scan, over a range of *m/z* 50–5000. High-resolution data were collected over a range of *m/z* 650–1500.

Results and Discussion

QCAL1, an artificial protein constructed using the QconCAT methodology [2–4], is a concatenation of 22 unique tryptic peptide sequences (Table 1) designed for the calibration, optimization, and comparison of a range of mass spectrometers. The QCAL1 peptides were additionally designed to assess and optimize instrument resolution, test the linearity of signal detection, and evaluate peptide separation by reversed-phase chromatography. As these peptides are incorporated into an artificial protein, it is also necessary for the user to validate common sample preparation procedures such as tryptic digestion and desalting. Characteristics are also incorporated within the design to assess peptide modification such as deamidation, methionine oxidation, and modification of lysine residues.

Peptide mass fingerprinting (PMF) experiments are typically performed using a matrix-assisted laser-desorption ionization (MALDI) time-of-flight (TOF) mass spectrometer for the identification of proteins following in-gel digestion with trypsin [5]. The peptides that are generated under these conditions and subsequently used for database searching, typically range between 500 and 3500 Da. However, MALDI-TOF instruments are usually calibrated with a mixture of synthetic peptides ≥900 Da, even though smaller peptides may enhance search algorithm scores and improve protein identification. QCAL1 was therefore designed to generate tryptic peptides with $[M + H]^+$ *m/z* values between ~400 and 3000. MALDI-TOF mass spectrometry of digested QCAL1 using a Voyager-DE STR (Figure 1a) demonstrates that up to 19 $[M + H]^+$ peptide ions can be used for calibration over the *m/z* range 400 to 3200. Similar data were also observed following MALDI analysis with an Ultraflex II TOF/TOF.

In MALDI-TOF MS, arginine-terminated tryptic peptides often give superior signals to their lysine-terminating counterparts [6]; however, peptide signals can be enhanced by conversion of lysine residues to homoarginine [7–9]. The almost identical peptides 8 and 11 (Table 1) were thus incorporated to test the efficiency of C-terminal homoarginine formation by lysine guanidination. MALDI-TOF spectra over *m/z* range 1410–1490, before (Figure 1b) and after (Figure 1c) guanidination are depicted, clearly indicating preferential detection of the lysine-terminating Q11 peptide after modification (Q11*). Based on the change in isotope distribution post-guanidination, base catalyzed deamidation of the peptides can also be observed [10].

Table 1. Sequence and function of QCAL1 peptides

Q-peptide	Sequence	Primary function	$[M+H]^+$: *m/z*	$[M+2H]^{2+}$: *m/z*	Relative ψ	Predicted retention time (min)	Actual retention time (min)
1	VFDEFKPLVEEPQNLIR	*m/z*, LC	2073.1015	1037.0544	35.98	40.0	39.3
2	VFDEFKPLVKPEEPQNLIR	*m/z*, resolution, LC	2298.2492	1149.6283	34.51	39.3	37.9
3	VFDEFKPLVKPEEKPQNLIR	*m/z*, resolution, LC	2426.3442	1213.6757	32.52	38.3	34.6
4	VFDEFKPLVKPEEKPQNKPLIR	*m/z*, resolution, LC	2651.4919	1326.2496	30.39	37.2	31.1
5	VFKPDEFKPLVKPEEKPQNKPLIR	*m/z*, resolution, LC	2876.6397	1438.8235	28.23	36.1	29.6
6	VFKPDEFKPLVKPEEKPQNKPLIKPR	*m/z*, resolution, LC	3101.7874	1551.3973	26.66	35.3	28.4
7	VFDEFQPLVEEPQNLIR	resolution (Q1)	2073.0651	1037.0368	37.90	41.0	40.1
8	GVNDNEEGFFSAR	calibration, linearity	1441.6342	721.3208	22.50	33.2	34.0
9	$[GGVNDNEEGFFSAR]_3$[a]	linearity of response	1498.6557	749.8315	23.20	33.6	33.8
10	$[GGGVNDNEEGFFSAR]_6$[b]	linearity of response	1555.6772	778.3422	22.92	33.5	33.7
11	GVNDNEEGFFSAK	guanidination (Q8)	1413.6281	707.3177	21.97	33.0	33.1
	GVNDNEEGFFSA[Har]	guanidination (Q8)	1455.6499	728.3286	–	–	–
12	AVMDDFAAFVEK	Met ox., LC	1342.6354	671.8216	36.14	40.1	39.8
13	AVMMDDFAAFVEK	Met ox., LC	1473.6758	737.3419	38.14	41.1	40.1
14	AVMMMDDFAAFVEK	Met ox., LC	1604.7163	802.8621	40.94	42.5	40.5
15	GLVK	*m/z*	416.2873	208.6476	10.07	27.0	N.D.
16	FVVPR	*m/z*, LC	617.3776	309.1927	16.98	30.5	30.9
17	ALELFR	*m/z*, LC	748.4358	374.7218	28.17	36.1	34.7
18	IGDYAGIK	*m/z*, LC	836.4518	418.7299	17.27	30.6	27.1
19	EALDFFAR	*m/z*, LC	968.4842	484.7460	31.77	37.9	38.7
20	YLGYLEQLLR	*m/z*, LC	1267.7051	634.3565	38.27	41.1	40.4
21	VLYPNDNFFEGK	*m/z*, LC	1442.6957	721.8518	29.19	36.6	38.3
22	LFTFHADICTLPDTEK	*m/z*, LC, Cys alkylation	1850.8999	925.9539	31.68	37.8	N.D.
	LFTFHADIC*TLPDTEK	*m/z*, LC, Cys alkylation	1907.9208	954.4640	–	–	38.4

Listed for each peptide is the sequence, primary reason for inclusion in QCAL1, the monoisotopic *m/z* ratio of the singly and doubly protonated peptide, the relative hydrophobicity (ψ) and retention time as predicted using the sequence-specific retention calculator algorithm [16] (http://hs2.proteome.ca/SSRCalc/SSRCalc.html), together with the actual retention time following reversed-phase chromatography. Each peptide is present as single copy except [a]Q9 with 3 copies and [b]Q10 with 6 copies. [Har] represents homoarginine. C* represents carbamidomethyl cysteine. N.D.: not detected.

Q21, previously overlapping with Q8, can also be clearly observed post-guanidination.

High-resolution Fourier Transform mass spectrometers, such as the ion cyclotron resonance (FT ICR) and Orbitrap instruments allow the determination of analyte masses to high accuracy (low or sub-ppm). Increasing use is therefore being made of these instruments in proteomics applications, primarily due to the reduction in false positive automated peptide identifications [11, 12]. Calibration of these instruments can be achieved using QCAL1 either as an external calibrant (Figure 2) (where average mass accuracy sub-1 ppm was achieved using the ICR instrument) or as an internal calibrant. To assess the resolving power of instruments such as these, QCAL1 incorporates peptides Q1 and Q7, representing a lysine to glutamine substitution, a difference of 0.0364 Da. Discriminating these two peptides requires an instrument resolution of ≥57,000 (FWHM), quite within the capabilities of both the instruments mentioned above. Data acquired on a 9.4T FT ICR mass spectrometer indicate that, as expected, these two peptides can be readily distinguished (Figure 2c), with peak resolution >105,000 (FWHM) being observed. Differentiation of these peptides can therefore be used as a benchmark for instrument resolution. In addition, deamidation of a number of tryptic peptides from QCAL1 was observed, with the amount of deamidation being dependent on sample preparation. The extent of deamidation can be adjusted by exposure of the peptides, rather than QCAL1, to high pH [13]. Detection of the deamidated form of $Q9^{2+}$ (and its discrimination relative to the native $Q9^{2+}$ first $[^{13}C]$ isotopomer peak) (Figure 2b) requires an instrument resolution >94,000 and can thus be used as an additional specification for standardizing the performance of high-resolution instruments.

Methionine oxidation is often observed as an artifact during sample preparation and it is thus difficult to discriminate artificially induced oxidation from post-translationally modified methionine. Peptides Q12, Q13, and Q14 were thus included to assess methionine oxidation as a result of sample handling. MALDI-TOF analysis of performic acid-induced oxidation of QCAL1 tryptic peptides demonstrated methionine sulfone formation on all three methionine containing peptides (data not shown), validating utility of these peptides to assess in vitro methionine oxidation.

Critical to the success of proteomics experiments and the characterization of peptides within complex mixtures is their separation by reversed-phase chromatography before mass spectrometric analysis. QCAL1 was therefore designed to incorporate peptides with a range of hydrophobicities, thereby permitting evaluation of reversed-phase chromatographic conditions for peptide separation. QCAL1 peptides demonstrated good chromatographic reso-

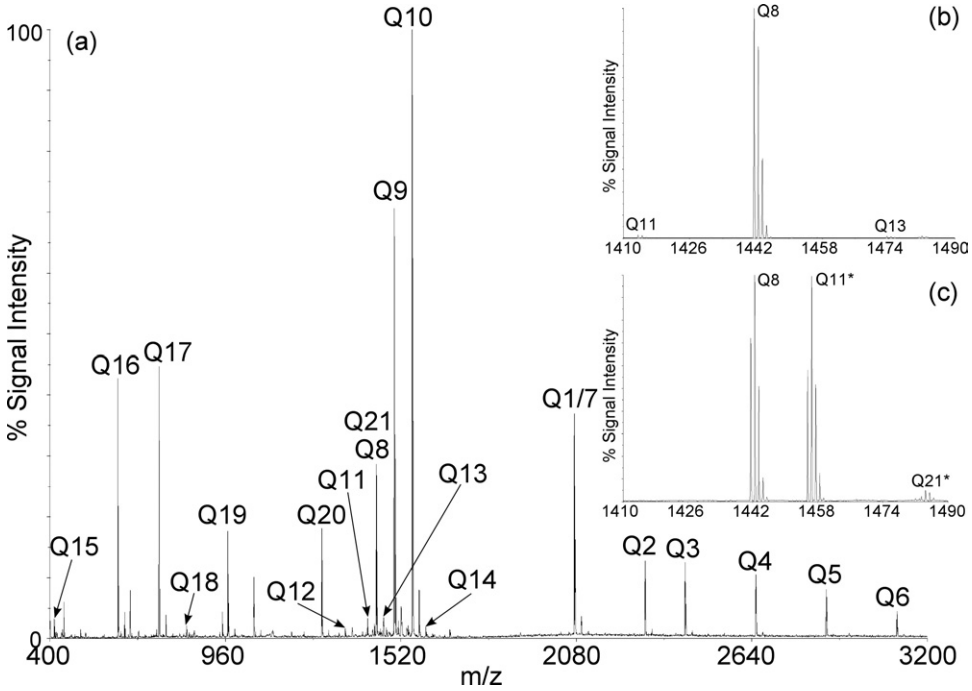

Figure 1. MALDI-TOF mass spectra of digested QCAL1. Limit peptides (the products of complete hydrolysis by the protease) generated after tryptic digestion of QCAL1 were analyzed following MALDI using a Voyager DE STR (**a**). Inset: trypsin hydrolysate of QCAL1 analyzed before guanidination (**b**) fails to differentiate Q8 and Q21. After guanidination (**c**) these peptides are clearly identifiable. Signal intensity of Q11 is also dramatically improved postguanidination.

lution, eluting between 5% and ~35% acetonitrile (Table 1, Figure 3), the typical range over which most tryptic peptides elute from C_{18} media [14, 15].

A significant number of laboratories use the [Glu^1]-fibrinopeptide B ("Glufib"; EGVNDNEEGFFSAR) to assess instrument sensitivity, and for calibration of

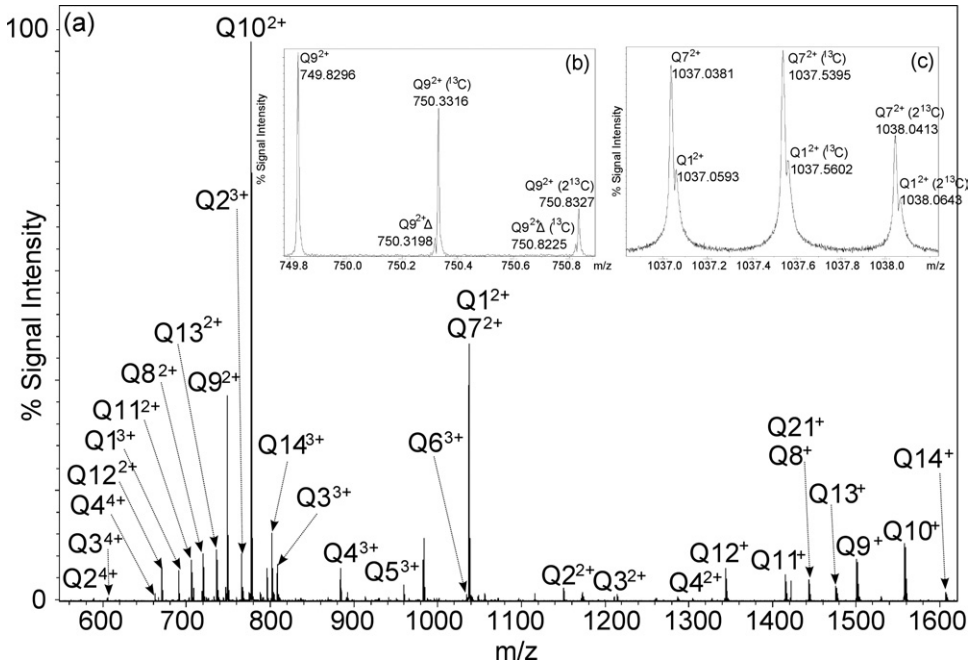

Figure 2. ESI FT ICR mass spectra of digested QCAL1 (**a**). Insets (**b**), (**c**) are high-resolution mass spectra collected over a range of *m/z* 650–1500, confirming that the resolution in this instrument mode is sufficient to readily detect peptide deamidation (**b**). In this case deamidated Q9 (Q9 Δ) is depicted. The doubly charged species of Q1 and Q7, separated by 0.0182 Th can also be readily differentiated (**c**).

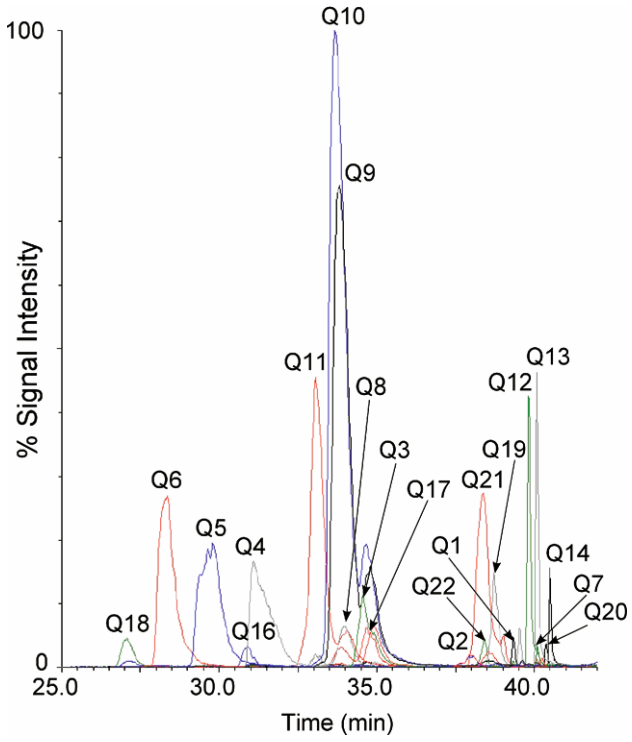

Figure 3. Analysis of digested QCAL1 by LC-MS/MS. Overlaid are the extracted ion chromatograms for the singly protonated species of Q16 (*m/z* of 617.4; peak at 30.9 min), Q17 (*m/z* of 748.4; peak at 34.7 min); doubly protonated species of Q7 (*m/z* of 1037.0; peak at 40.1 min), Q8 (*m/z* of 721.3; peak at 34.0 min), Q9 (*m/z* of 749.8; peak at 33.8 min), Q10 (*m/z* of 778.3; peak at 33.7 min), Q11 (*m/z* of 707.3; peak at 33.1 min), Q12 (*m/z* of 671.8; peak at 39.8 min), Q13 (*m/z* of 737.34; peak at 40.1 min), Q14 (*m/z* of 802.9; peak at 40.5 min), Q18 (*m/z* of 418.7; peak at 27.1 min), Q19 (*m/z* of 484.7; peak at 38.7 min), Q20 (*m/z* of 634.4; peak at 40.4 min), Q21 (*m/z* of 721.9; peak at 38.3 min); triply protonated species of Q1 (*m/z* of 691.7; peak at 39.3 min); quadruply charged species of Q2 (*m/z* of 575.3; peak at 37.9 min), Q3 (*m/z* of 607.3; peak at 34.6 min), Q22 (*m/z* of 636.6; peak at 38.4 min); $[M + 5H]^{5+}$ of Q4 (*m/z* of 531.1; peak at 31.1 min) and Q5 (*m/z* of 576.1; peak at 29.6 min); $[M + 6H]^{6+}$ of Q6 (*m/z* of 517.8; peak at 28.4 min). All peptides elute between 5% and ~35% acetonitrile with a maximal peak width of ~40 s, average peak width of ~20 s.

fragment ion *m/z* following collision-induced dissociation. However, this sometimes requires a different instrument set-up (for example, analyte infusion) than is used for peptide analysis by LC-MS, and instrument sensitivity determined after positional optimization of the ionization needle used for infusion cannot readily be compared with instrument sensitivity for LC-MS analysis, if a different front-end configuration is used. A modified version of the Glu-fibrinogen peptide sequence (where the amino-terminal Glu was removed to decrease the potential of missed tryptic cleavage) was therefore incorporated into QCAL1 (Q8) to permit calibration post-fragmentation (data not shown) and assessment of instrument sensitivity using the same front-end configuration as is used for proteomics studies. The range of *m/z* and charge states of the peptides included in QCAL1 (Q1-Q7) also permits optimization of the *m/z*-dependent nature of the collision voltage offset required to obtain high quality tandem MS spectra and thus the best possible peptide identification.

For quantification studies, assessing the linearity of signal detection of the instrument is also critical. Multiple copies of two variants of the Glu-fibrinogen derived peptide, where one (Q9, three copies) or two (Q10, six copies) additional glycine residues have been added to the peptide amino-terminus, were thus included in QCAL1 (Table 1). Analysis of these three peptides by LC-MS on a quadrupole-time of flight (Q-TOF) instrument demonstrated good linearity of signal, with Q8:Q9:Q10 being detected at a ratio of 1.0:3.1:6.2 ($n = 4$, with S.D. of the ratios being 0.06 and 0.18, respectively), calculated following integration of the extracted ion chromatogram for each of the peptides. This observation is consistent with the hypothesis that the additional glycine residues have little impact on peptide response factors in this system. The small percentage of the deamidated forms of these peptides did not compromise the linearity of response. MALDI-TOF (ABI Voyager instrument) analysis of the same peptides (Figure 1) yielded a ratio of 1.0:2.6:4.0 ($n = 11$, with S.D. of the ratios being 0.28 and 0.53, respectively). This may suggest either nonlinearity of response due to signal suppression of peptide ions in the unfractionated mixture, or that the additional glycine residues compromise ionization by MALDI to an extent not apparent by ESI. A similar effect was also seen following analysis on the Bruker Daltonics Ultraflex II TOF/TOF (data not shown) with ratios of 1.0:2.6:4.7 ($n = 6$, with S.D. of the ratios being 0.10 and 0.41, respectively).

Conclusions

QCAL1 can be used for calibration and optimization of a number of instruments widely used in proteomics studies, as well as for testing and comparison during the development of new techniques and instruments for peptide analysis. This standard—available in substantial amounts with continuing availability due to the nature of its production—will enable the proteomics community to define in more detail the behavior of the instruments used in large-scale studies, thus facilitating long-term reproducibility in proteomics projects. Future iterations of QCAL could assess additional features used in proteomics analysis, for example peptide separation by strong-cation exchange chromatography, or alternative proteolytic agents such as AspN or GluC. Concatamers could also be designed to assess more extensively the performance of high-resolution instruments, or optimize different fragmentation techniques (collision-induced dissociation versus electron-transfer dissociation). The concept of "designer" proteins, such as QCAL, is thus readily extended from absolute quantification to new applications that could generate an effectively unlimited resource to ensure that all laboratories adopt common, carefully defined baseline calibration and assessment materials.

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