The use of internal standards for macromolecule quantification by LC-MS:

lessons learned from small molecule assays

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- Internal standards for small molecules
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- Intact protein internal standards
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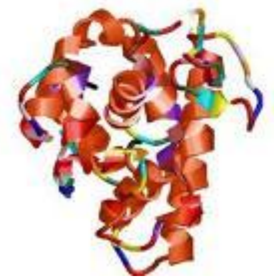

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Introduction

Why macromolecules with LC-MS?

- does not require raising of antibodies
- gives structural information
- provides improved accuracy and precision
- results in better comparability between labs

Important role of internal standards

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Introduction

Internal standard

A substance added in a constant amount to all samples (calibration, quality control and unknown) to correct for experimental variability during sample analysis.

A substance with an analytical behaviour very similar to the analyte, but which gives a signal that can be distinguished from that of the analyte.

For optimal correction, it is added as early as possible during the analytical procedure.

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Small molecules

Typical analytical procedure:

- Pipetting of sample
- Addition of internal standard
- Extraction
- (Derivatization)
- Chromatography
- Detection

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Small molecules

Internal standard corrects for

Variability in
- Extraction behaviour
- Derivatization yield
- Transfer and injection volumes
- Chromatographic performance
- Detection

Internal standard does not correct for

Variability in
- Initial sample aliquoting
- Peak integration

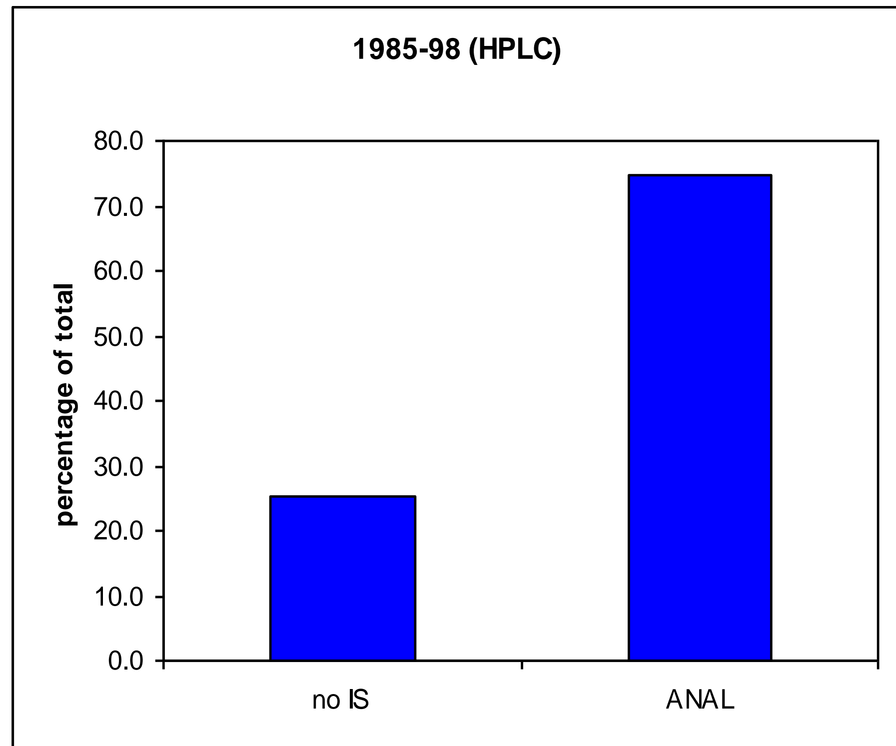
Small molecules

LC-UV methods

Internal standard:

- None
- Structural analogue

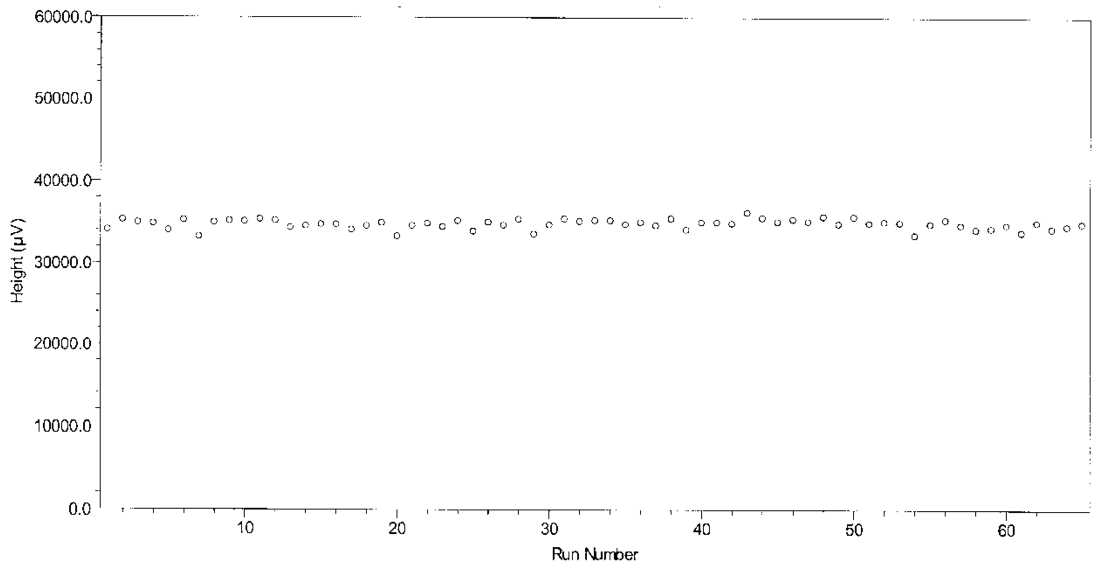
Typically no more than one



Small molecules

LC-UV methods

Typical IS plot
(SPE-LC-UV)
LLOQ 15 ng/ml



Experimental variability usually under control

Small molecules

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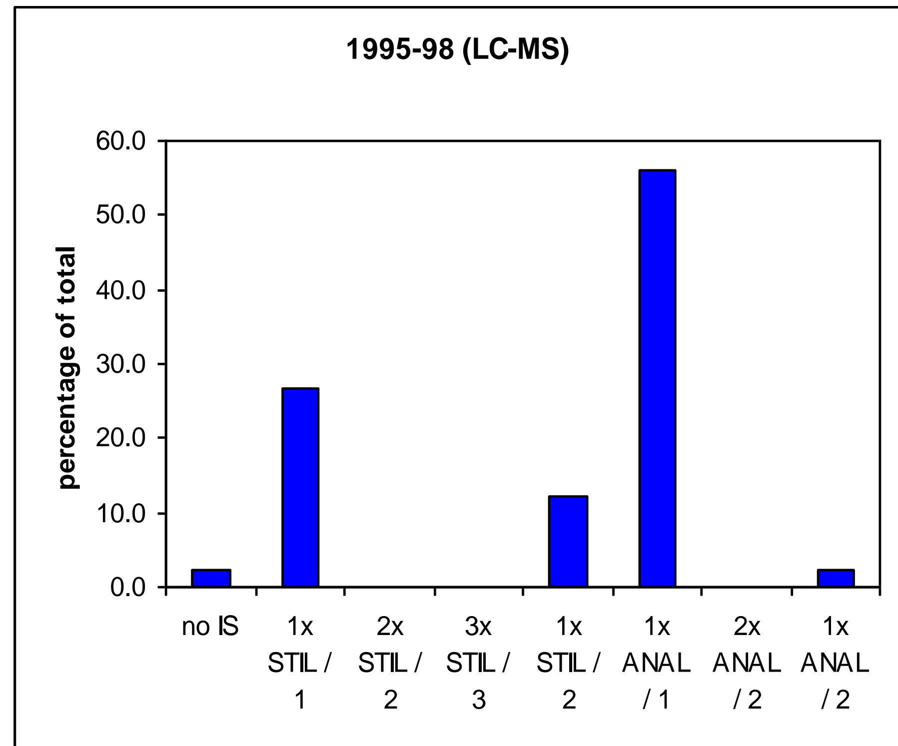
LC-MS methods

Internal standard:

- None
- Structural analogue
- Stable-isotope form

Typically one for each analyte



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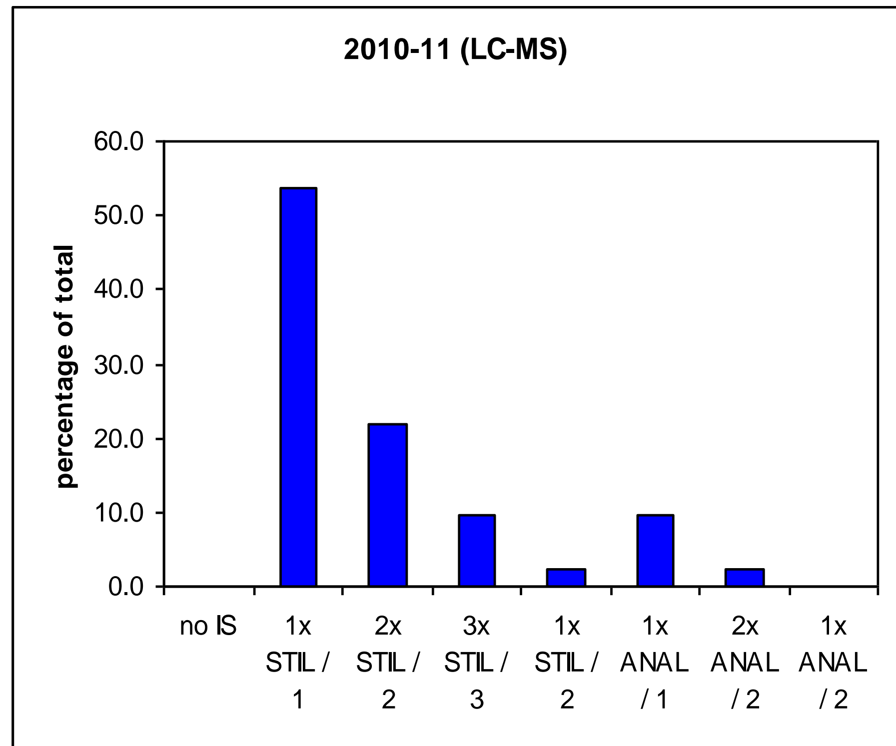
Small molecules

LC-MS methods

Internal standard:

- None
- Structural analogue
- Stable-isotope form

Typically one for each analyte



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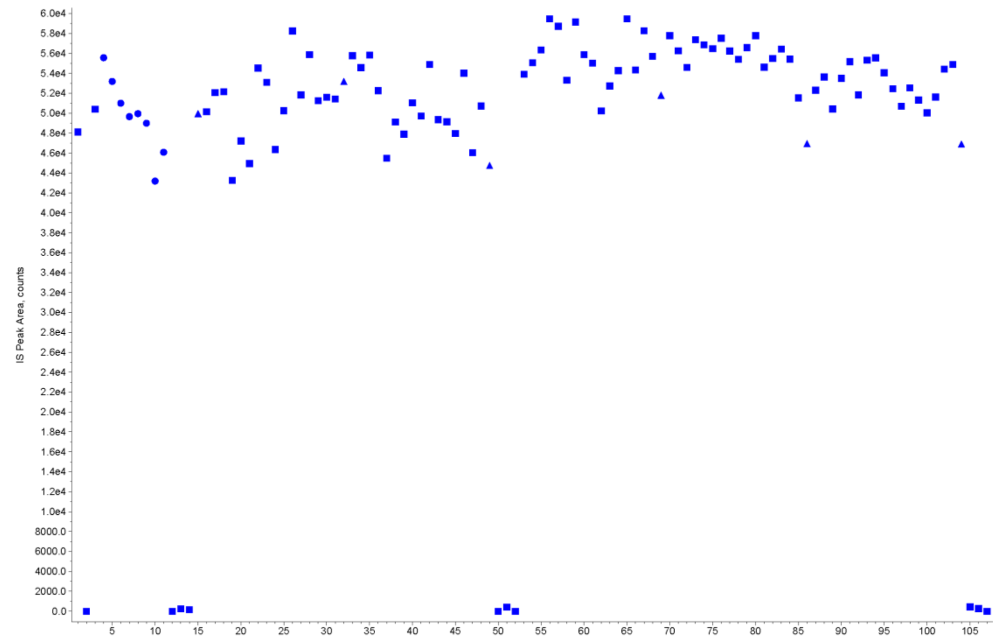
Small molecules

LC-MS methods

Typical IS plot
(SPE-derivatization-
LC-MS/MS)
LLOQ 50 pg/ml



Variability from detection process,
internal standard crucial for LC-MS

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Macromolecules

Typical analytical procedure:

- Pipetting of sample
- Addition of internal standard
- (Extraction of macromolecule)
- Digestion
- Extraction of signature peptide
- (Derivatization)
- Chromatography
- Detection

Macromolecules

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Internal standard corrects for

Variability in

- Extraction behaviour (if co-extracted)
- Digestion efficiency (if co-digested)
- Derivatization yield
- Transfer and injection volumes
- Chromatographic performance
- Detection

Macromolecules

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Internal standard does not correct for

Variability in

- Initial sample aliquotting
- Extraction behaviour (if not co-extracted)
- Digestion efficiency (if not co-digested)
- Peak integration

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Macromolecules

LC-MS methods

Internal standard:

- None
- Stable-isotope form of protein
- Structural analogue of protein
- Stable-isotope form of peptide
- Structural analogue of peptide
- Stable-isotope form of extended peptide
- Peptide derivatized with stable-isotope labeled reagent

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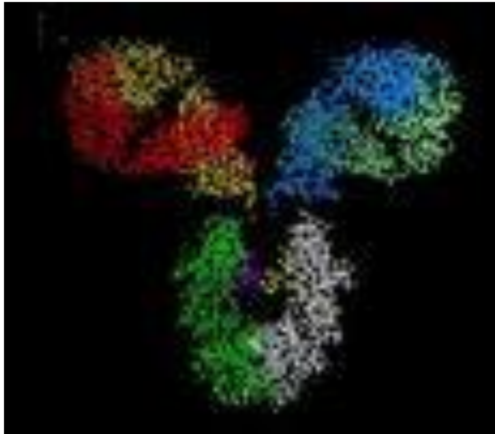
Protein internal standards

Stable-isotope labeled protein



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Protein internal standards

Stable-isotope labeled protein

Biosynthesis by intact cells on culture medium containing

- a ^{13}C-^{15}N-labeled amino acid
- ^{13}C-labeled glucose and/or ^{15}N-labelled ammonium

Cell-free synthesis using supernatant of e.g. *E. coli* lysate, containing protein synthesis machinery and addition of

- a mixture of amino acids among which one or more labeled ones
- energy regenerating system (ATP)
- DNA template

G.W. Becker, Briefings Funct. Gen. Proteom. 7 (2008) 371-382

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Protein internal standards

Stable-isotope labeled protein

Advantages:

- optimal correction for all extraction steps (including immuno-affinity extraction)
- optimal correction for digestion
- yields stable-isotope form of signature peptide, thus optimal correction for peptide extraction and MS detection

Disadvantages:

- not readily available
- expensive

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Protein internal standards

Stable-isotope labeled protein

An example:

Therapeutic monoclonal antibody in marmoset serum (pre-clinical analysis)

	Analyte	Internal standard
protein:	146 761 Da (100 Thr)	147 257 Da (100 Thr-$^{13}C_4$-^{15}N)
sign. peptide:	2202 Da (4 Thr)	2222 Da (4 Thr-$^{13}C_4$-^{15}N)
fragment:	780 Da (2 Thr)	790 Da (2 Thr-$^{13}C_4$-^{15}N)

O. Heudi et al., Anal. Chem., 80 (2008) 4200-07.

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Protein internal standards

Stable-isotope labeled protein

- Addition of internal standard
- Reduction and alkylation
- Overnight tryptic digestion of whole serum
- (cation-exchange) SPE of digest
- LC-MS/MS of signature peptide
- LLOQ 5 µg/ml
- bias<10%
- CV<15% (<20% at LLOQ)

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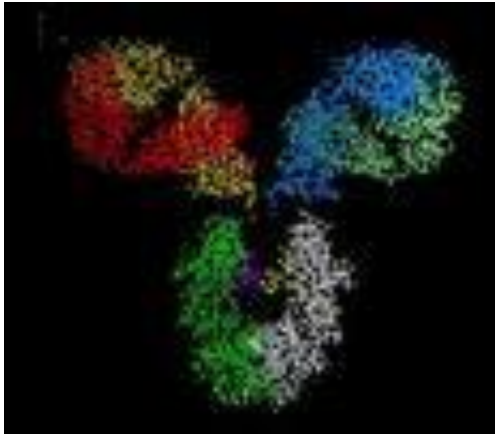
Protein internal standards

Other protein



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Protein internal standards

Other protein

Obtained from commercial sources

Advantages:

- typically good availability, inexpensive
- correction for digestion

Disadvantages:

- yields structural analogue of signature peptide, thus no optimal correction for peptide extraction and MS detection
- (depending on structure) no optimal correction for protein extraction

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Protein internal standards

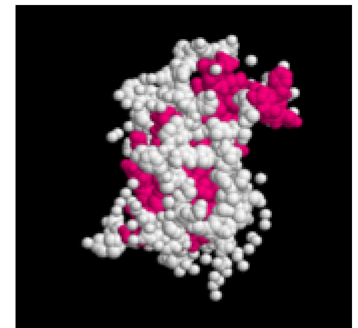
Other protein

An example:

Somatropin in human plasma, using bovine fetuin as internal standard (€150 / gram)

	Analyte	Internal standard
protein:	22 124 Da	48 400 Da
sign. peptide:	976 Da (LFDNAMLR)	1267 Da (QDGQFSVLFTK)
fragment:	718 Da	626 Da

Z. Yang et al., Anal. Chem., 79 (2007) 9294-9301



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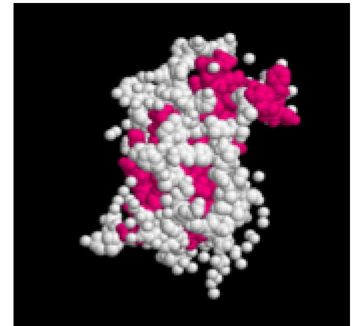
Protein internal standards

Other protein

- Addition of internal standard
- Reduction and alkylation
- Overnight digestion of whole plasma
- 2D (reversed-phase/cation-exchange) SPE of digest
- LC-MS/MS of signature peptide
- LLOQ 1 µg/ml
- no precision / accuracy data



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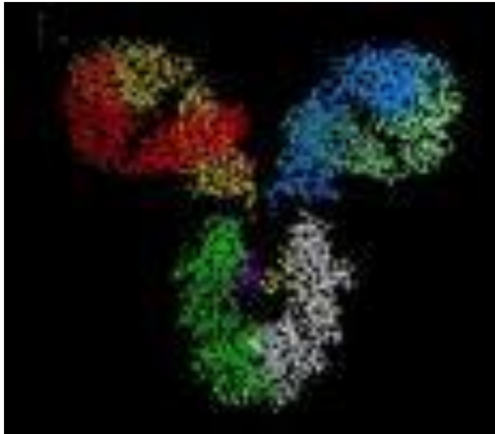
Peptide internal standards

Stable-isotope labeled peptide



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Peptide internal standards

Stable-isotope labeled peptide

Chemical (solid-phase) synthesis using one or more isotope-labeled amino acids

Advantages:

- typically good availability, relatively inexpensive
- optimal correction for peptide extraction and MS detection

Disadvantages:

- no correction for protein extraction
- no correction for digestion

Peptide internal standards

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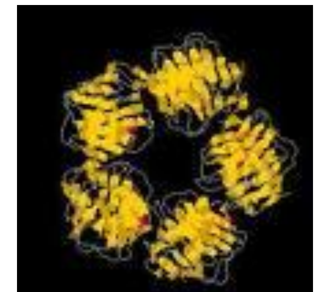
Stable-isotope labeled peptide

An example:

C-reactive protein in rat urine

	Analyte	Internal standard
protein:	25 106 Da	n.a.
sign. peptide:	1524 Da (TSFNEILFWTR)	1530 Da (TSFNEIL*FWTR)

M. Aguiar et al., Anal. Biochem., 354 (2006) 175-181.



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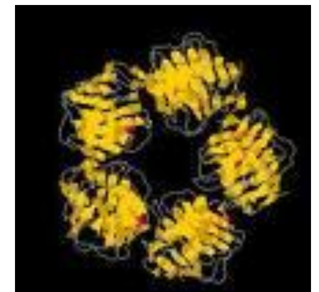
Peptide internal standards

Stable-isotope labeled peptide

- Addition of internal standard
- Reduction and alkylation
- Overnight digestion of urine
- LC-MS (TOF) of signature peptide
- Analysis against calibration curve of unlabeled peptide!
- LLOQ 50 ng/ml peptide (0.8 µg/ml protein)
- Bias<15%
- CV<5%



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Peptide internal standards

Comparison stable-isotope labeled peptide and unlabeled intact protein

Analyte: therapeutic human mAB in rat serum

Sign. peptide GLEWSVSGISYSGSNTHYADSVK

Internal standard 1: bovine fetuin

Sign. peptide TPIVGQPSIPGGPVR CV: 6-9%

Internal standard 2:

GLEWSVSGISYSGSNTHYADSV*K CV: 7-13%

Z. Yang et al., Anal. Chem., 79 (2007) 9294-9301

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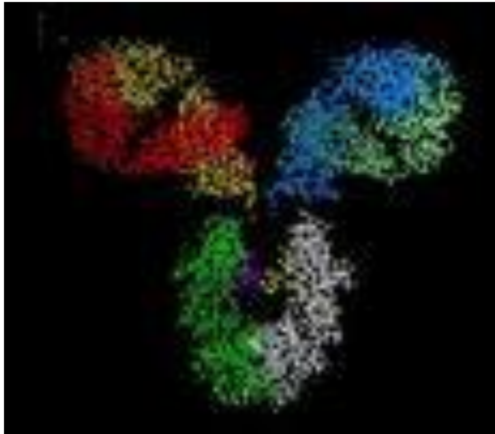
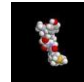
Peptide internal standards

Other peptide





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Peptide internal standards

Other peptide

Obtained from commercial sources

Advantages:

- good availability, inexpensive

Disadvantages:

- no correction for protein extraction
- no correction for digestion
- no optimal correction for peptide extraction and MS detection

Peptide internal standards

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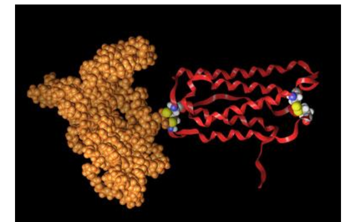
Other peptide

An example:

Pegylated therapeutic protein in monkey plasma

	Analyte	Internal standard (peptide)	(protein)
protein:	ca 50 000 Da	n.a.	ca 50 000 Da
sign. peptide:	1104 Da (EIPISINYR)	1020 Da (L*LIYFTSR)	1268 Da (DYQPISINYR)
fragment:	432 Da	234 Da	863 Da



S.T. Wu et al., Rapid Commun. Mass Spectrom. 25 (2011) 281-290.

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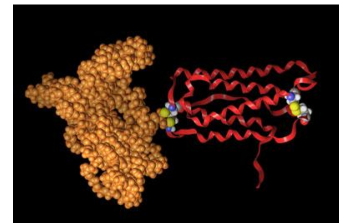
Peptide internal standards

Other peptide

- Addition of protein internal standard
- Extraction with isopropanol
- Addition of peptide internal standard
- Overnight tryptic digestion
- LC-MS/MS of signature peptide
- LLOQ 10 ng/ml
- Bias<15% (peptide IS)
- Bias<12% (protein IS)
- CV<12% (<17% at LLOQ) (peptide IS)
- CV<11% (protein IS)



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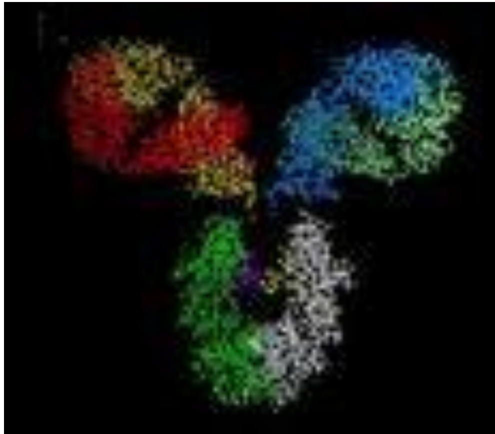
Peptide internal standards

Stable-isotope form of extended peptide



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Peptide internal standards

Stable-isotope form of extended peptide

Obtained from commercial sources

Advantages:

- good availability, relatively inexpensive
- optimal correction for peptide extraction and MS detection
- some correction for digestion

Disadvantages:

- no correction for protein extraction

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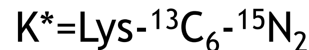
Peptide internal standards

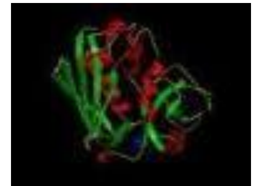
Stable-isotope form of extended peptide

An example:

Staphylococcus enterotoxin TSST-1 in drinking water

	Analyte	Internal standard
protein:	ca 22 000 Da	4928 Da (HQLTQIHGLYR*- LPTPIELPLK*- NTDGSISLIIFPSPYYSPAFTK*)
sign. peptide:	1120 Da (LPTPIELPLK)	1128 Da (LPTPIELPLK*)





V. Brun et al., Mol. Cell. Proteomics 6 (2007) 2139-2149.

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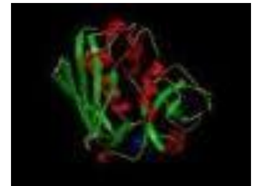
Peptide internal standards

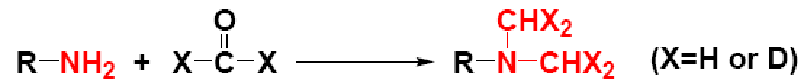
Stable-isotope form of extended peptide

- Addition of internal standard
- Overnight tryptic digestion
- LC-MS (QTOF) of signature peptide
- no calibration curve, LLOQ ~0.1 ng/ml
- digestion efficiency IS only 33% of that of analyte



Differential labeling

Derivatization of signature peptide with unlabeled reagent (analyte) or labeled reagent (internal standard)



Advantages:

- good availability, inexpensive
- optimal correction for MS detection

Disadvantages:

- no correction for protein extraction
- no correction for digestion
- no correction for peptide extraction

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Differential labeling

An example:

Therapeutic monoclonal antibody in monkey serum

protein:	ca 150 000 Da
sign. peptide:	1174 Da (NWPLTFGGGTK)
analyte:	1234 Da $(CH_3)_2$-NWPLTFGGGTK-$(CH_3)_2$
analyte fragment:	905 Da
internal standard:	1242 Da $(CHD_2)_2$-NWPLTFGGGTK-$(CHD_2)_2$
internal standard fragment:	909 Da Da

C. Ji et al., Anal. Chem., 81 (2009) 9321-9328.

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Differential labeling

Serum:

- Denaturation, reduction, alkylation
- Overnight tryptic digestion
- labeling with formaldehyde (2 hours)

Pure analyte solution:

- Denaturation, reduction, alkylation
- Overnight tryptic digestion
- labeling with d_2-formaldehyde (2 hours)

- mix solutions
- LC-MS/MS of derivatized signature peptide
- LLOQ 1 µg/ml
- Bias<10%, CV<15%

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Comparison

Internal standard	Covers protein extraction	Covers digestion	Covers peptide extraction	Covers MS	Availability
Labelled protein	+	+	+	+	-
Other protein	+/-	+/-	+/-	+/-	+/-
Labelled peptide	-	-	+	+	+
Other peptide	-	-	+/-	+/-	+
Labelled extended peptide	-	+/-	+	+	+
Differential labelling	-	-	-	+	+

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