Potential of hyphenated MS for Cr speciation analysis: An insight into the safety of Cr-enriched food/supplements

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Outline

- Chromium (Cr) and Cr species in Cr dietary sources: Essentiality versus toxicity
- Chromium food supplements: Focus on organically-bound Cr yeast
- Development of reference methodology and materials for food Cr speciation: EU requirements and challenges
- Application to:
  - Speciation of inorganic and organically-bound Cr in Cr-enriched yeast supplements
  - Provision of values for CrVI to a PT water sample

Chromium and food supplementation

Chromium speciation in food and supplements – why?

- Chromium (III) is an essential trace element involved in lipid and glucose metabolism
- Chromium (VI) is highly toxic and shown to be carcinogenic
- It is usually considered that almost all* the chromium in food is present as chromium (III)
- About 0.5-1% of chromium (III) present in the normal diet is absorbed (depending on the diet)
- Concerns about the safety of dietary Cr (III), which might need to be re-evaluated since it was found to cause DNA damage *in vivo***

*** Health Protection Agency – Chromium Toxicological Overview, UK, 2007**
**** European Food Safety Authority Journal, 2009, 1112, 1-20**

Chromium from the diet

- Most of ingested chromium is transformed to Cr(III) in the stomach

- Average Cr burden (UK, Adults*) ~ 150 μg day^{-1}
  - From food: daily intake of Cr adult 117 μg day^{-1}
    - From drinking water < 10 μg day^{-1} (2 L day^{-1} basis)
    - Inhalation – estimated as 60 ng day^{-1}
- Main dietary sources of Cr
  - Fish
  - Meat
  - Fruits
  - Sugar
  - Food supplements

*** Health Protection Agency – Chromium Toxicological Overview, UK, 2007**

The need for reliable Cr speciation data

- Cr(VI) causes numerous toxicological effects
- Chromium (VI) compounds are positive in the majority of *in-vitro* mutagenicity tests reported, also affects the respiratory, cardiovascular, gastrointestinal, hepatic, renal, and neurological systems
- Cr(III) easily oxidizes to Cr(VI), and vice versa
- Cr – from essential to toxic at the same concentration!
- **Reliable speciation data and therefore, reference methods needed!**

Strategic approach to the speciation of Cr in food supplements

- Bioavailable forms of Cr compensate for deficiency in the Northern European diet and insulin resistance in some population groups

- Chromium supplements represent ~6% of current mineral supplement sales
  - Cr supplements on the market are poorly characterised and in some cases their food safety has been questioned
  - Cr picolinate or nicotinate is approved by European authorities but Cr-enriched yeast is often used because of better bioavailability

- During fermentation yeast produces a range of organic Cr-containing compounds of unknown composition
  - Cr may be mainly present as Cr(III) but evidence is needed
  - Regulated max. 0.2% Cr(VI) from the total Cr, provided that total Cr < 250 μg/day*

*** EFSA Journal, 2012, 10, 2951**

Measurement strategy

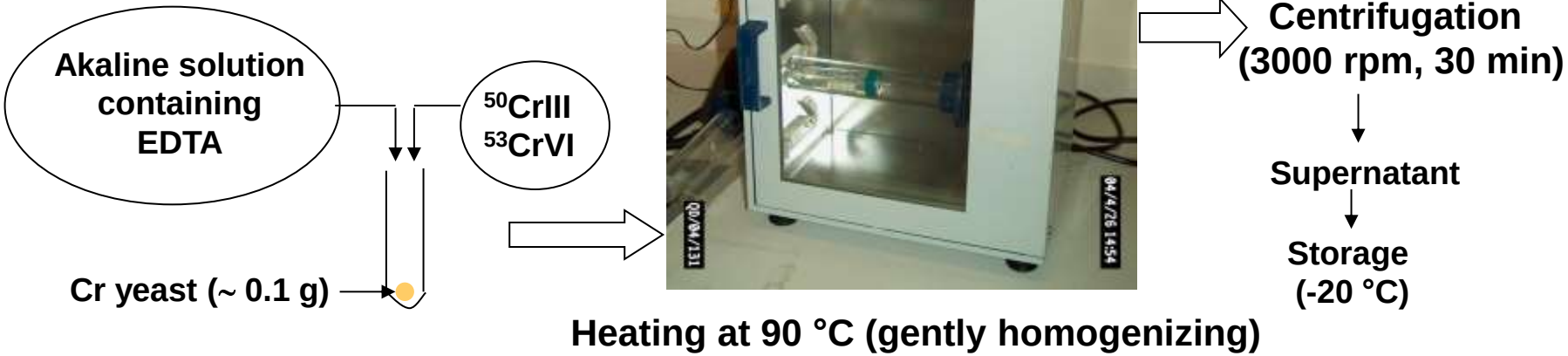
- Variety of extraction methodologies for Cr species will be used (e.g. accelerated solvent extraction, microwave-enhanced enzymatic/alkaline hydrolysis)

- Collision-reaction cell ICP-MS in helium mode will be used to minimise the effects of spectral interferences affecting the detection of Cr isotopes (e.g. $^{40}Ar^{12}C$ on ^{52}Cr and $^{37}Cl^{16}O$ on ^{53}Cr)

- Size-exclusion followed by reversed phase, hydrophilic interaction or anion-exchange LC in combination with ICP-MS and ESI MS/MS for monitoring multi-element species distribution and for identification of Cr species in supplement extracts

- Species-specific isotope dilution (IDMS) quantitation of inorganic Cr species using reversed phase HPLC-ICP-MS after alkaline hydrolysis

Single species-specific (SS) IDMS schematic



Main measurement challenges for speciated chromium supplements

- The diversity of Cr species present at different concentrations in a complex ‘solid’ matrix

- The lack of pure calibration standards, spikes and reference materials, needed for method validation
  - Only NIST SRMs and BCR CRMs for inorganic Cr in solutions, soil/waste and air particulates are available
  - No speciated Cr CRM in a complex dietary supplement has been produced
  - No Cr spikes for inorganic species IDMS are commercially available

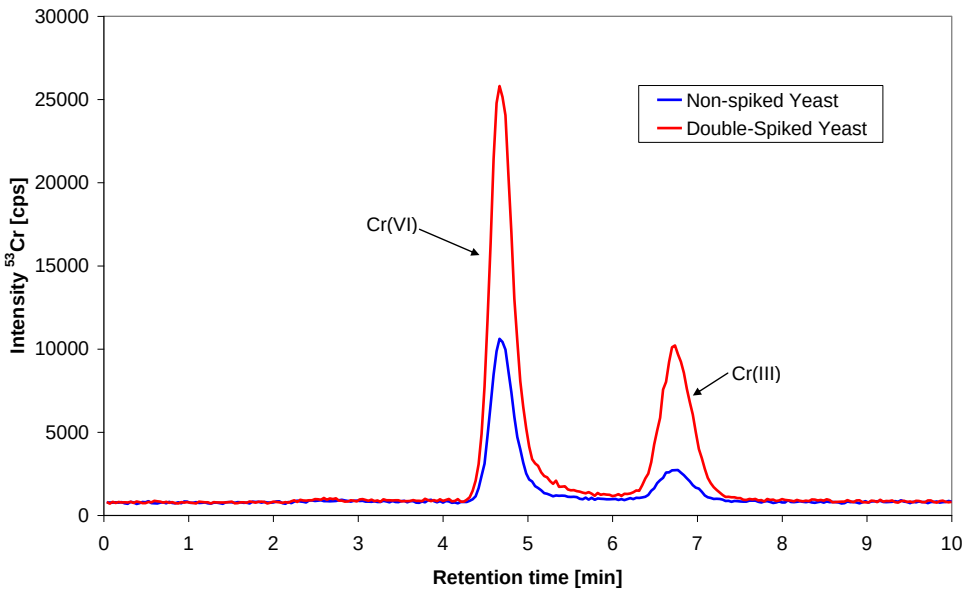
- Lack of isotopically labelled spikes required for species specific IDMS of organo-chromium compounds

Methodology for the determination of inorganic chromium species

Ion-pair/Chelation Reversed Phase HPLC-ICP-MS:

- Mobile phase – 0.18 mM TPABr, 1 mM EDTA, pH 8.5 (isocratic)
- Sample diluent – 2.8 mM TPABr, 4 mM EDTA, pH 8.5
- Analytical column PEEK PLRP-S 100Å column (macroporous styrene/divinylbenzene (PS/DVB))
- Quantification of Cr(VI) and Cr(III) species by species specific IDMS in food supplements
- Quantification of Cr(VI) and Cr(III) species by external calibration in water samples

Determination of Cr(III) and Cr(VI) in cellular-bound Cr(III) enriched yeast with SS-IDMS calibration



> 68% of Cr(III) converts to Cr(VI); basic media

> Some Cr(VI) converts to Cr(III); reacting with the yeast

> Conversion of Cr(III) to Cr(VI) could lead to the wrong conclusion (suggesting that Cr(VI) is present at µg/g levels)

	Amount of Yeast (g)	Cr(III) concentration (n=3) (µg/g)	Recovery Cr(III) STD (%)
Cr enriched Yeast 1	0.050	327 ± 12	84 ± 5
Cr enriched Yeast 2	**0.100**	**318 ± 5**	**101 ± 2**

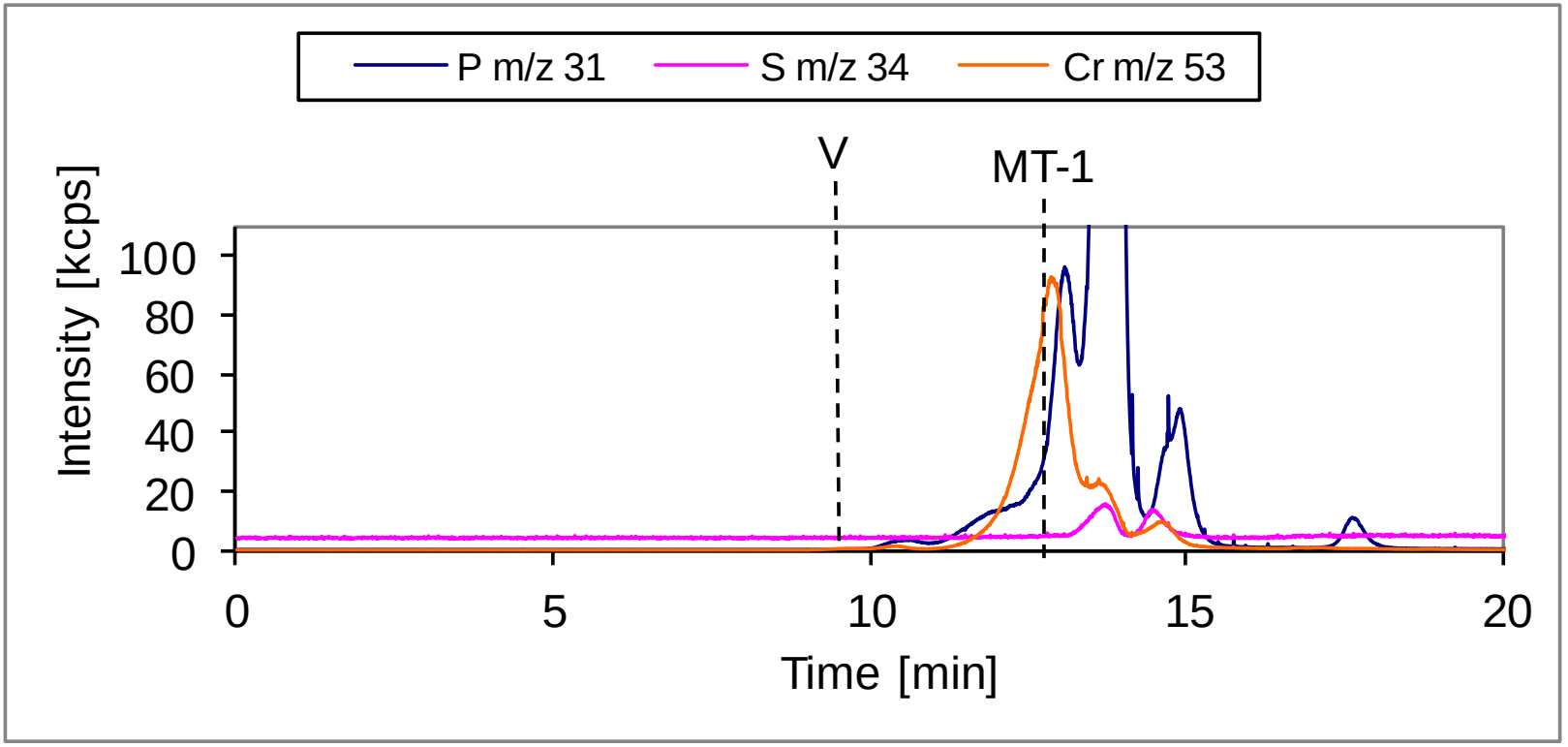
Sequential Cr extraction of cellular-bound Cr(III) enriched yeast

Steps	Amount of extractable Cr (µg/g, n=6)	% Ext eff
1. Water soluble	70 ± 4	25.4
2. Driselase	78 ± 5	28.2
3. SDS	118 ± 5	42.7
4. Protease	1± 0.5	0.25
5. Unextractable	1.5 ± 1	0.52

Total Cr content of Cr enriched yeast 276 ± 3 µg/g, n=8
sum of extractions results in an mean recovery of 97.1 ± 2.1% n=6

SEC-ICP-MS speciation of the water extractable chromium in yeast

TSKgel G3000 PWXL; 10 mM ammonium acetate, pH 6.5



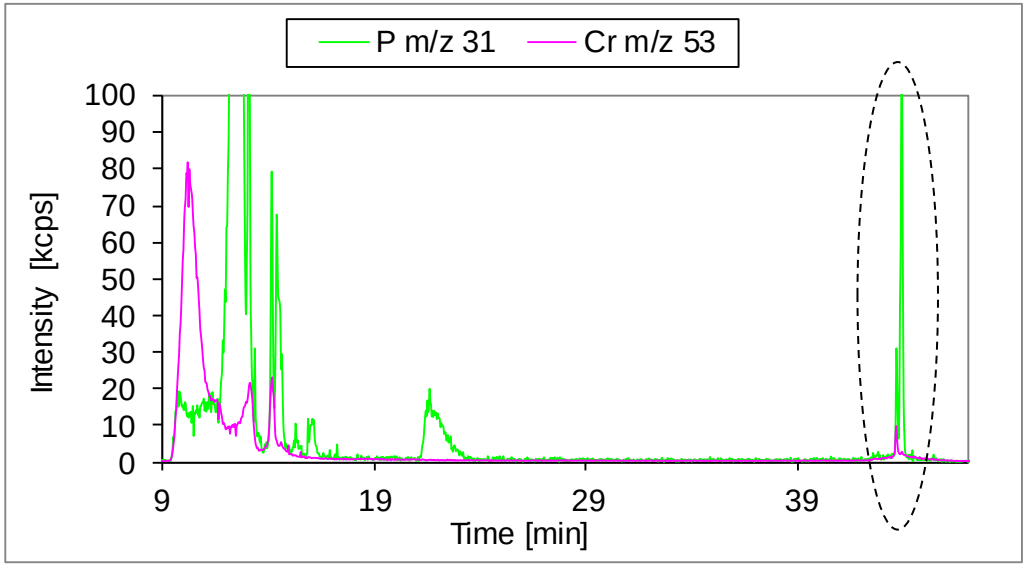
V – column void volume; MT1 – metallothionein 1 (10kDa)

Cr associated with a fraction with MW < 10 kDa

Reversed phase HPLC-ICP-MS profile for a water extract from Cr-enriched yeast

Chromatographic conditions

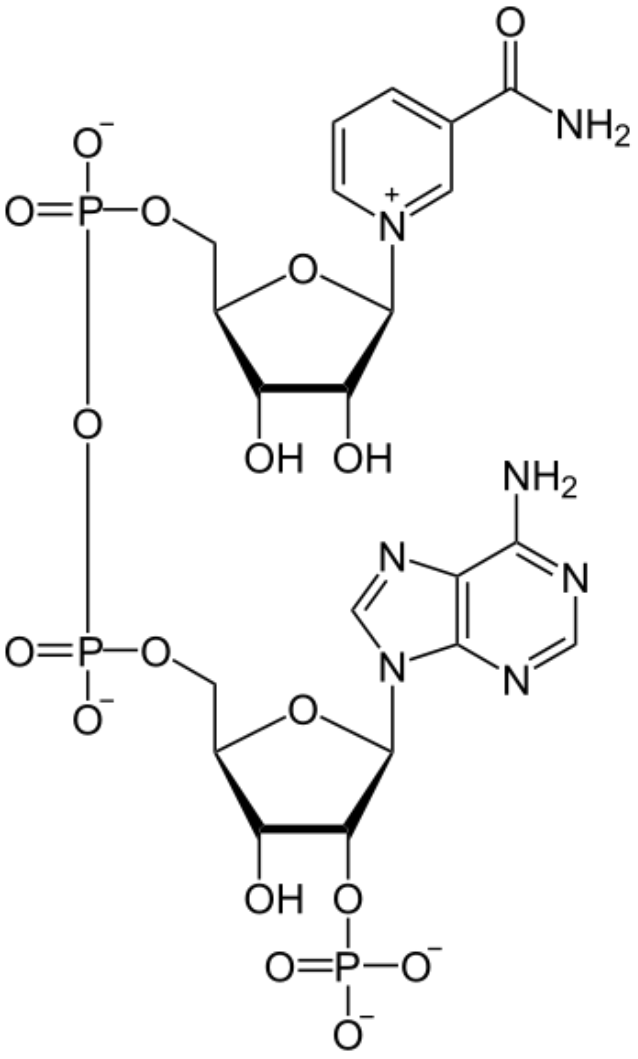
Chromatography columns	ZORBAX RX-C8, 4.6x250mm, 5µm followed by COSMOSIL 5PYE Waters 2.0x150mm
Mobile phase	A) methanol : 1mM ammonium acetate 80 : 20% (v/v) B) 1mM ammonium acetate pH 6.5
Gradient time min 0 20 30 33 35 70	 1 % (A) 99% (B) 1 % (A) 99% (B) 90% (A) 10% (B) 90% (A) 10% (B) 1% (A) 99% (B) 1% (A) 99% (B)
Injection volume (µL)	20
Column temperature (˚C)	20
Flow (mL/min)	0.20



- **Variety of Cr-containing species**
- **P detected in several peaks**
- **Peak with Rt ~44 min contains both P and Cr**

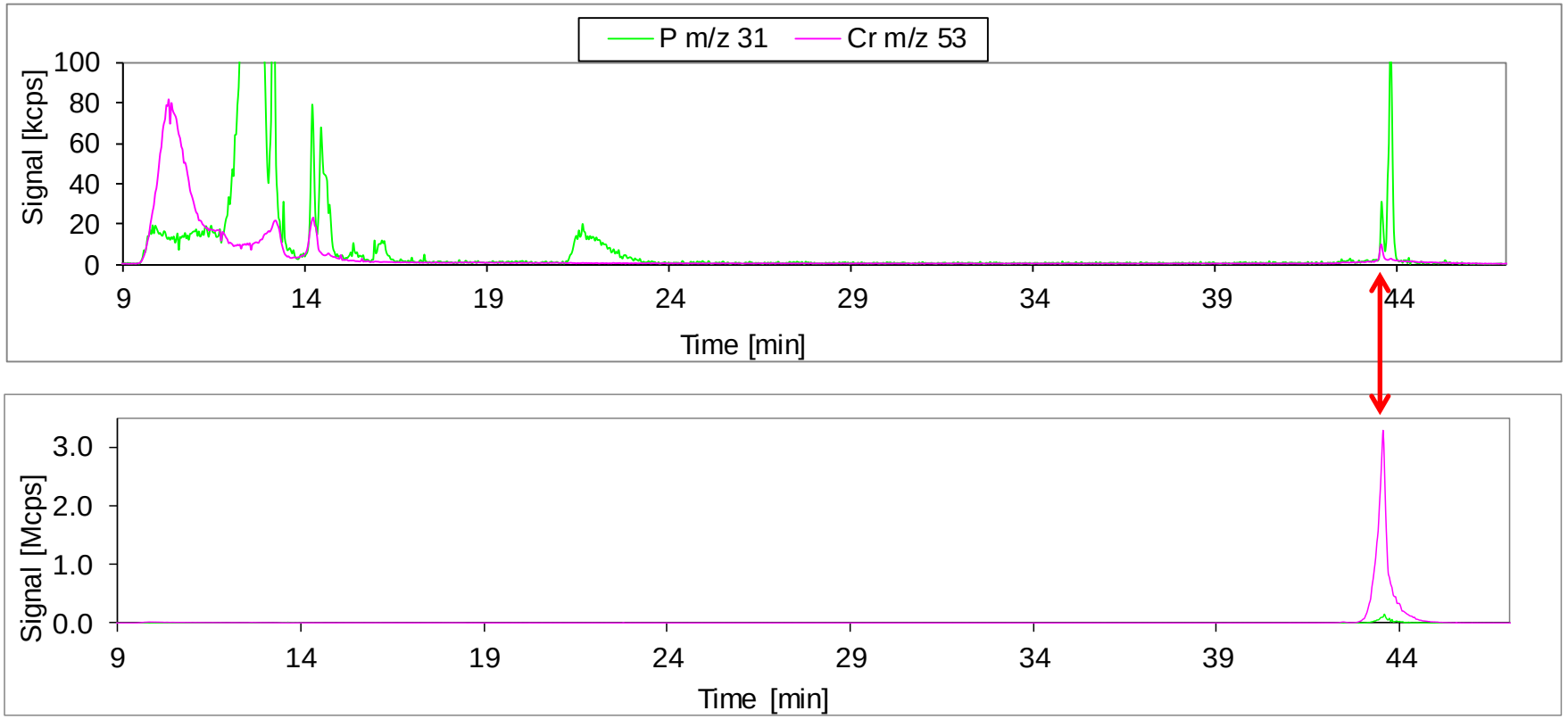
NADP+ and synthesis of the chromium-NADP complex

- Nicotinamide adenine dinucleotide phosphate, abbreviated NADP+ is a coenzyme used in lipid and nucleic acid synthesis
- Polar compound, widely present in cells
- From chemical point of view it is polydentate ligand (N, O, C, PO_n donor) with chelating properties
- Literature indications of possible complexation with Cr*



*** M. Beran, R. Stahl, M. Beran (Jr), Analyst. 120 (1995), 979-981.**

Chromatograms of a Cr-enriched yeast water extract and synthesized Cr-NADP complex solution

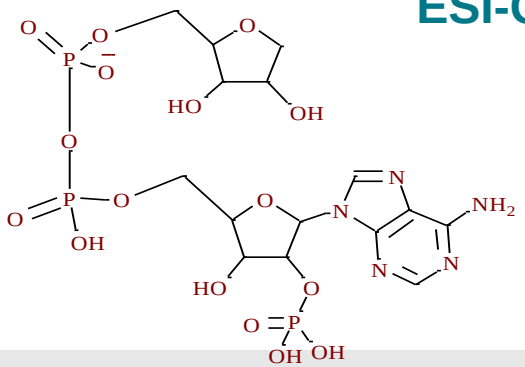


- ICP-MS retention time matching

Reversed phase HPLC/ESI-Q-TOF-MS analyses of Cr-NADP complex and yeast water extract

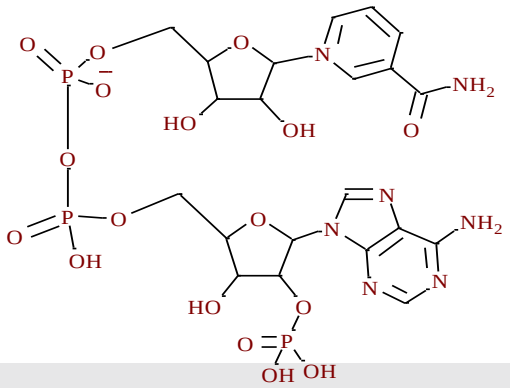
Structure	Formula	Measured Mass	Mass Accuracy (ppm)
A+B+Water	$C_{36}H_{46}N_{12}O_{34}P_6Cr_2$	737.9693	-0.3
A+B+2Water	$C_{36}H_{48}N_{12}O_{35}P_6Cr_2$	746.9778	-4.3
B+B+Water	$C_{42}H_{52}N_{14}O_{35}P_6Cr_2$	798.9950	-2.1
B+B+2Water	$C_{42}H_{54}N_{14}O_{36}P_6Cr_2$	808.0017	-3.84

Ligand A



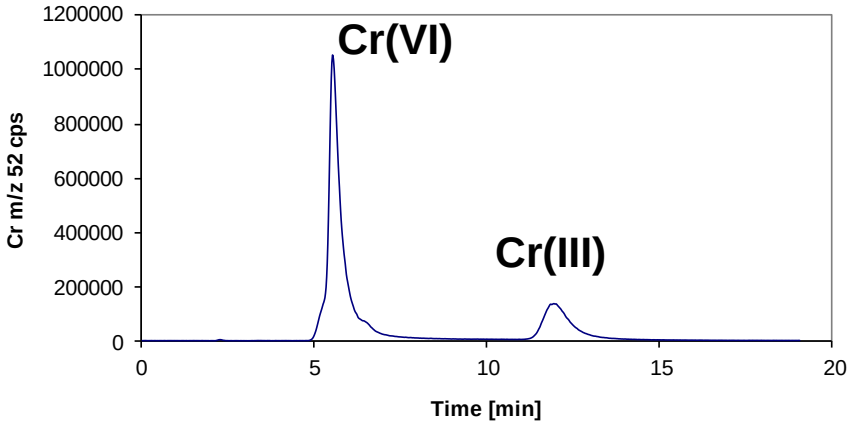
Agilent LC/MS 6530 Accurate-Mass ESI-Q-TOF-MS (negative ionisation)

Ligand B



Determination of Cr(III) and Cr(VI) in clean and waste waters by HPLC-ICP-MS with external calibration

- AQUACHECK proficiency testing scheme solutions – clean and waste waters:



Spike recoveries:

Cr(VI) 85 ng g^{-1}
recovery 103.9 ± 2.2 %

Cr(III) 25 ng g^{-1}
recovery 98.8 ± 3.3 %

Identification Round	^{52}Cr(VI) (µg L^{-1})	^{52}Cr(III) (µg L^{-1})	Assigned Value, uncertainty and acceptable range Cr(VI) (µg L^{-1})	Recovery % Cr(VI)
433-1	4.46±0.01	<0.07	4.58±0.018 (3.58-5.58)	97.4 ± 0.3
441-1	10.38±0.11	<0.07	10.37±0.045 (8.30-12.44)	100.1 ± 1.1

Summary and outlook

- The use of methodology based on species-specific isotope dilution mass spectrometry has been proven essential to obtain reliable data for CrIII and CrVI from solid samples e.g. food supplements
- For liquid samples (e.g. waters), the use of external calibration strategies may fit for purpose, if sufficient evidence for no redox species interconversion during sample dilution/analysis is provided
- The IDMS reference methodology developed and validated is currently in use for the characterisation of first “speciated” Cr-yeast reference material
- ICP-MS is an essential tool to elemental species identification (provides retention time elemental information for data mining), however,
- Highly selective LC and molecular MS detectors are invaluable tools to provide MS to-the-end accurate, required for the identification of metallometabolites in complex bio-samples (e.g. NADP-Cr in Cr-yeast)

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National Measurement System

National Measurement Office

NPL National Physical Laboratory

LGC

nel technology for life