This document is confidential and is proprietary to the American Chemical Society and its authors. Do not copy or disclose without written permission. If you have received this item in error, notify the sender and delete all copies.

Cobalamin concentrations in fetal liver show gender differences: a result from using an HPLC-ICP-MS as an ultra-trace cobalt speciation method

Journal:	*Analytical Chemistry*
Manuscript ID	ac-2016-03730w.R1
Manuscript Type:	Article
Date Submitted by the Author:	17-Nov-2016

Complete List of Authors:	Bosle, Janine; University of Aberdeen, Chemistry Goetz, Sven; University of Aberdeen, Chemistry Raab, Andrea; University of Aberdeen, Department of Chemistry Krupp, Eva; University of Aberdeen, Chemistry Scheckel, Kirk; US EPA, ORD-NRMRL-LRCPD-WMB Lombi, Enzo; University of South Australia, Centre for Environmental Risk Assessment and Remediation Meharg, Andrew; Queen's University Belfast, Institute for Global Food Security Fowler, Paul; University of Aberdeen, Centre for Reproductive Endocrinology & Medicine Feldmann, Joerg; University of Aberdeen, Chemistry

SCHOLARONE™
Manuscripts

Cobalamin concentrations in fetal liver show gender differences: a result from using an HPLC-ICP-MS as an ultra-trace cobalt speciation method

Janine Bosle[1], Sven Goetz[1], Andrea Raab[1], Eva M. Krupp[1], Kirk G. Scheckel[2], Enzo Lombi[3], Andrew A. Meharg[4], Paul A. Fowler[5] and Jörg Feldmann[*1]

[1]TESLA (Trace Element Speciation Laboratory), Department of Chemistry, Meston Walk, University of Aberdeen, Aberdeen, AB24 3UE, Scotland, UK

[2]U. S. Environmental Protection Agency, National Risk Management Research Laboratory Land Remediation and Pollution Control Division, 5995 Center Hill Avenue, Cincinnati, OH, USA

[3]Future Industries Institute, University of South Australia Building X, Mawson Lakes Campus Mawson Lakes, SA-5095, Australia

[4]Institute for Global Food Security, Queen's University Belfast, Malone Rd, Belfast BT9 5BN, UK

[5]Institute of Medical Sciences, School of Medicine, Medical Sciences & Nutrition, University of Aberdeen, Aberdeen AB25 2ZD, UK

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

22 ***ABSTRACT***

23

24 Maternal diet and lifestyle choices may affect placental transfer of cobalamin (Cbl) to
25 the fetus. Fetal liver concentration of Cbl reflects nutritional status with regards to
26 vitamin B12, but at these low concentration current Cbl measurement methods lack
27 robustness.
28 An analytical method based on enzymatic extraction with subsequent RP-HPLC
29 separation and parallel ICP-MS and ESI-Orbitrap-MS to determine specifically Cbl
30 species in liver samples of only 10-50 mg was developed using 14 pig livers.
31 Subsequently 55 human fetal livers were analyzed. HPLC-ICP-MS analysis for cobalt
32 (Co) and Cbl gave detection limits of 0.18 ng/g and 0.88 ng/g d.m. in liver samples
33 respectively with a recovery of >95%. Total Co (Co_t) concentration did not reflect the
34 amount of Cbl or vitamin B12 in the liver. Cbl bound Co contributes only 45 +/- 15 %
35 to Co_t. XRF mapping and μXANES analysis confirmed the occurrence of non-Cbl
36 cobalt in pig liver hot spots indicating particular Co. No correlations of total cobalt
37 nor Cbl with fetal weight or weeks of gestation were found for the human fetal livers.
38 Although no gender difference could be identified for total Co concentration, female
39 livers were significantly higher in Cbl concentration (24.1 +/- 7.8 ng/g) than those
40 from male fetuses (19.8 +/- 7.1 ng/g) (p=0.04). This HPLC-ICP-MS method was able
41 to quantify total Co_t and Cbl in fetus liver and it was sensitive and precise enough to
42 identify this gender difference.

43

44

INTRODUCTION

Vitamin B12 or cobalamin (Cbl) is an essential vitamin stored in the liver.[1] Cbl can occur in different molecular forms of which only two, methyl-cobalamin (Me-Cbl) and adenosylcobalamin (Ado-Cbl), are physiologically active. Me-Cbl is a cofactor for enzymes in the carbon-1 metabolism, while Ado-Cbl is a cofactor for enzymes involved in 1,2 H-shifts and transfer of an electronegative group to the neighboring carbon atom. Although the majority of vitamin B12 is stored in the liver, the concentrations are at the ultra-trace level.[2] The methods routinely used to determine vitamin B12 status are either based on microbiological or immunoenzymatic determination of Cbl in serum and have been criticized for their overestimation or failure to determine low levels of Cbl and their lack of precision.[3,4] Analytical methods for Cbl determination based on chromatography coupled to ICP-MS or ESI-MS have been reported but so far mainly used for food-supplements.[5,6,7,8] An analytical method based on thermal acidic denaturation with liquid/liquid extraction of beef liver with subsequent RP-HPLC separation of the main four Cbl species with ESI-MS detection showed promising results for the determination of Cbl in liver samples.[9] The sensitivity and specificity of this method though needs improving due to the small size of tissue samples available from the human fetus and the extraction needs to be confirmed by using a complementary direct speciation method such as EXAFS and XANES.[10]

The wider aim of this study was to develop a method for Cbl quantitation which is robust but sensitive enough to detect low background levels in liver samples. This method was then applied to human fetal liver samples to identify whether vitamin B12 status varies with body weight, gestation age or gender.

This was achieved by the following objectives:

- To test whether it was possible to determine quantitatively the physiologically active forms of Cbl (Me-Cbl and Ado-Cbl) besides cyano-cobalamin (CN-Cbl), and hydroxyl-cobalamin (HO-Cbl) individually when spiked to liver in order to evaluate the full conversion of those active forms into CN-Cbl.
- To evaluate the quantitative extraction and determination of vitamin B12 and if possible the physiologically active forms of Me-Cbl and Ado-Cbl from pig

liver samples using HPLC with parallel detection of using ICP-MS for Co and ESI-MS for the individual Cbl forms.

- To confirm whether extraction was altering the Cbl content by using a direct speciation method for which no extraction is necessary by XRF (X-ray fluorescence) mapping with subsequent µXANES (x-ray absorption near edge spectroscopy).
- To apply the protocol to 55 human fetal livers and quantify the Cbl concentration and Co_t concentration in fetal livers and evaluate the results with regards to liver weight, sex and gestation age.

EXPERIMENTAL SECTION

Chemicals and Material

Water used throughout the experiments was of 18 MΩ cm quality (Elga, UK). The different cobalamin (Cbl) standards, methyl-cobalamin (Me-Cbl), hydroxy-cobalamin (HO-Cbl), adenosyl-cobalamin (Ado-Abl) and cyano-cobalamin (CN-Cbl) (98%, Sigma-Aldrich Germany) were dissolved in water at a concentration of 1 mg Co/mL and stored in the dark. The eluents for the HPLC were 0.1% formic acid (Analytical reagent grade, Fisher Scientific UK) in water (eluent A) and 0.1% formic acid in methanol (HPLC grade S, Rathburn UK) (eluent B). Co standards (High purity standards, UK) for calibration were prepared based on a stock solution of 1000 mg Co/L diluted with 1% HNO_3, conc. (supra pure, BDH UK). A Rh solution (Specpure, Alfa Aesar Germany) served as internal standard. For the sample preparation different organic solvents were used, including methanol (Laboratory reagent grade, Fisher Scientific UK) and acetone (Laboratory reagent grade, Fisher Scientific UK). For the liver extraction acetate buffer (pH 5) (acetic acid: extra pure, Sigma-Aldrich Germany), Papain (from Carica Papaya, Sigma Aldrich Germany), potassium cyanide (Fisher Scientific UK) and HCl (Laboratory reagent grade, Fisher Scientific UK) were used. Nitric acid conc and hydrogen peroxide (Laboratory reagent grade, Fisher Scientific UK) were used for the microwave-assisted digestion of liver samples prior to total Co measurements by ICP-MS.

Pig Liver samples

For the method development 14 pig liver samples were used as a proxy for the human liver samples. The pig livers were bought at a local butcher in Aberdeen and stored at -20°C before analysis.

Human fetal liver

The collection of fetal material was approved by the NHS Grampian Research Ethics Committees (REC 04/S0802/21). Women seeking elective, medical terminations of pregnancy were recruited with full written, informed consent by nurses working independently at Aberdeen Pregnancy Counseling Service. Only fetuses from normally-progressing pregnancies (determined by ultrasound scan), from women over 16 years of age with a good grasp of English and between 11-21 weeks of gestation, were collected.

Fetuses were transported to the laboratory within 30 minutes of delivery, weighed, crown-rump length recorded, and sexed. Livers were snap-frozen in liquid nitrogen and stored at -85°C. All morphological data were from the same study as published in Drake et al.[11] and are summarized in **Table 1.**

Table 1: morphological data for mothers and fetuses (mean ± SE)

	Females	**Males**
Maternal age (yrs)	25.0 ± 1.1	23.3 ± 1.2
Maternal BMI (m^2/kg)	24.6 ± 1.1	25.5 ± 0.9
N	25	30
Fetal weight (g)	122.4 ± 19.3	68.6 ± 11.1
Fetal crown-rump length (mm)	111.2 ± 6.3	95.1 ± 4.5
Fetal age (weeks of gestation)	15.7 ± 0.6	14.1 ± 0.3

Experiments and methods

Optimization of extraction method

ACS Paragon Plus Environment

All solutions were handled under dim light conditions. Cbl was extracted from homogenized freeze dried liver samples using a method modified from Rappazzo et al. (*12*). To 50 mg liver 10 μL potassium cyanide solution (1 % w/v), 500 μL buffer (50 mol L^{-1} sodium acetate pH 5.0) and varying amounts of papain were added. In the optimized final method 5 mg papain was added. The solutions were incubated at different temperatures and for different time periods and centrifuged after cooling in order to optimize the extraction efficiency with regards to total Cbl. The supernatant was stored at 4°C in the dark until analysis.

Total cobalt (Co_t) determination

20-50 mg pig or human liver samples were weighed accurately in duplicate into plastic centrifuge tubes. Subsequently 2.0 mL HNO_3 were added and left to stand overnight at 25°C. Hydrogen peroxide (0.5 mL) and 0.250 mL of 20 mg/kg rhodium as internal standard were added and the samples digested in a Mars 5 microwave oven (Matthews Inc, USA). Blanks as well as the listed CRMs were digested in every round of samples as well. Samples were cooled and diluted with deionised water to a final concentration of 2% (v/v) nitric acid. Cobalt was measured by high-resolution ICP-MS (Element 2, Thermo Fisher Scientific) at *m/z* 59 at low resolution (R = 300) to gain more sensitivity in addition to m/z 103 for rhodium as the internal standard.

Cobalt speciation using HPLC-ICP-MS/ESI-MS

The separation and determination of the 4 cobalamin species was carried out by coupling a HPLC to an ESI-MS and ICP-MS using a reversed phase column with a methanol gradient program. The ESI-MS was used in positive FTMS-mode of species identification. The instrumental parameters are listed in **Table S1**. To allow quantification the HPLC-ESI-MS was also linked to an ICP-MS. The HPLC flow was split before the UV-detector with a ratio of 3:1 (ESI-MS: ICP-MS), the continuous internal standard (Rh) used for ICP-MS was added via a T-piece before the ICP-MS nebulizer to correct for matrix changes. Parameters are listed in **Table S1** and further description of the split set up can be found by Bluemlein and co-workers.[13]

Synchrotron XRF mapping and μXANES speciation of Cobalt

Synchrotron based X-ray fluorescence (XRF) was used for mapping Co distribution in shredded freeze-dried pig liver samples. The samples were prepared as thin pressed pellets. Elemental maps were collected at beamline 20-ID (PNC/XOR) at the Advanced Photon Source (APS), Argonne National Laboratory.

The electron storage ring operated at 7 GeV. A nitrogen cooled Si(111) double crystal monochromator, calibrated using a cobalt metal foil, was used to generate the X-ray beam. The fluorescence signal was collected using a 13-element Ge detector (Canberra). Four maps of (1.5 x 1.5mm) were obtained by rastering the sample through the 9700 eV beam of 10 x 6 μm with a step size of 20 μm and an integration time of 0.3 s/step. The elemental mapping of trace levels of Co in a high Fe matrix (hemoglobin) is challenging due to the large overlap between the Fe Kβ emission line (7,059 eV) and the Co Kα emission line (6,915 eV). Therefore, a script was developed to subtract the contribution of the Fe Kβ signal from the sum of the Fe Kβ plus Co Kα signal based on the known ratio of Fe Kβ relative to the collected Fe Kα signal. Areas on the maps, corrected for Fe interference, showing accumulation of Co were investigated using μXANES in order to confirm the identification of Co and assess its speciation. Three scans per point of interest were collected, averaged and normalized using Athena.[14] The spectra obtained were compared to cobalt standards of vitamin B12 (CN-Cbl), coenzyme B12 (Ado-Cbl), methylcobalamin (Me-Cbl), hydroxycobalamin (HO-Cbl) and also Co^{+I} and Co^{+II} salts.

Quality controls and statistics

Blanks as well as CRMs were measured with every batch of the liver digests for total hepatic Co analysis. Certified standard reference materials (NIST RM8415, NRC TORT-2) were used to check reproducibility and accuracy, with both better than +/- 5 %. Spiking experiments into the liver sample of Cbl-species were performed to evaluate the integrity of the Cbl species and the accuracy and precision of the Cbl-determination.

Statistical analyses of data were performed using JMP 9.0.2 software (Thomas Learning, London, UK). For method development ANOVA two way tests were performed. For the human liver samples the normality of data distribution was tested with the Shapiro-Wilk test and non-normally distributed data were log-transformed and re-checked for normality prior to analysis by ANOVA and Tukey-Kramer HSD

and *t*-tests, where data were not normalized, or the variances remained unequal, non-parametric tests were performed (Wilcoxon Test).

Safety

Work with cyanide poses an extra level of risk, which needs to be assessed before starting to work. Especially cyanide should not be poured in acidic solution below pH 5 to prevent the generation of volatile HCN.

RESULTS AND DISCUSSION

Separation and detection

A mixture of the 4 Cbl standards in water was measured by HPLC-ICP-MS. The 4 Cbl species were baseline separated on a C8 column with a methanol gradient. All were well retained and separated within 14 min and were detected by their Co signal on m/z 59 by ICP-MS and simultaneously by their molecular peaks $[M+H]^+$ and $[M+2H]^{2+}$ by ESI-MS (**Figure 1a-b**). It can be seen that the ICP-MS Co response did not change significantly during the chromatographic run although a gradient program was used (**Figure S1**). This behavior is in contrast to what has been observed for arsenic or Sulphur.[15] Co does not benefit from the carbon enhancement effect, as does As or Se, since it is already fully ionized in the plasma. The response factor for the Orbitrap varied considerably depending on species as indicated by the different peak heights (**Figure 1a**). Using the elemental calibration (**Figure S1a-b**) the amount of cobalt can be calculated for each species using the ICP-MS signal, whereas when solely the ESI-MS is used an individual calibration curve for every Cbl is required. For quantification an external calibration was used with Co element standards (Co^{2+}) from 1 to 100 µg Co/L using the ICP-MS signal. The calculated detection limit for aqueous solutions is about 0.05 µg Co/L based on 3 times standard deviation of the background noise. This is more sensitive than the methods listed in a recent review.[2]

Stability of the standards over time

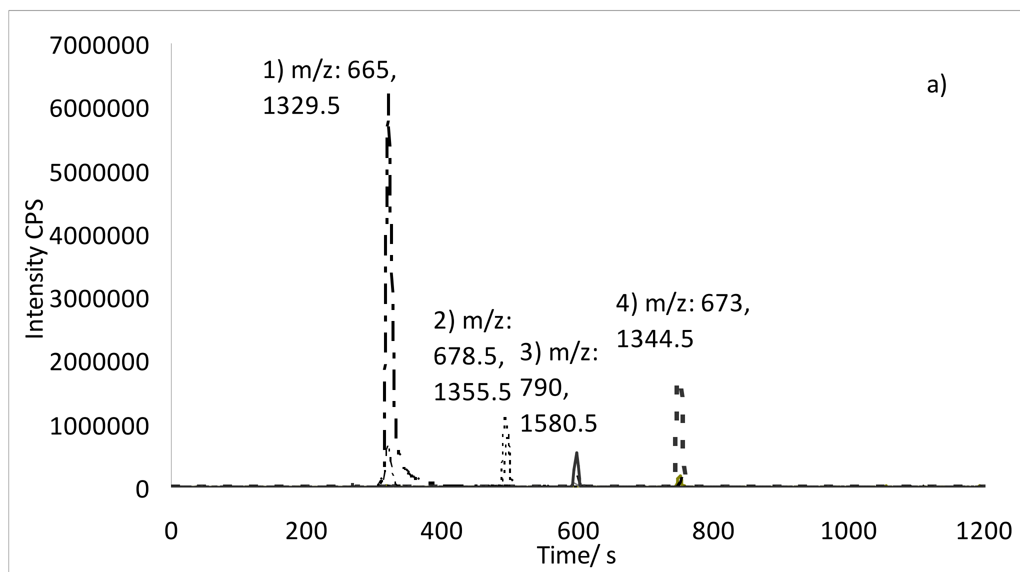
In order to assess the stability of cobalamins (objective 1), a comparison between freshly prepared and stored (frozen) solutions was performed (**Figure 1b and 1c).** When the standards were stored for more than a day in a freezer, species transformation took place. CN-Cbl was stable, while Me-Cbl and Ado-Cbl showed

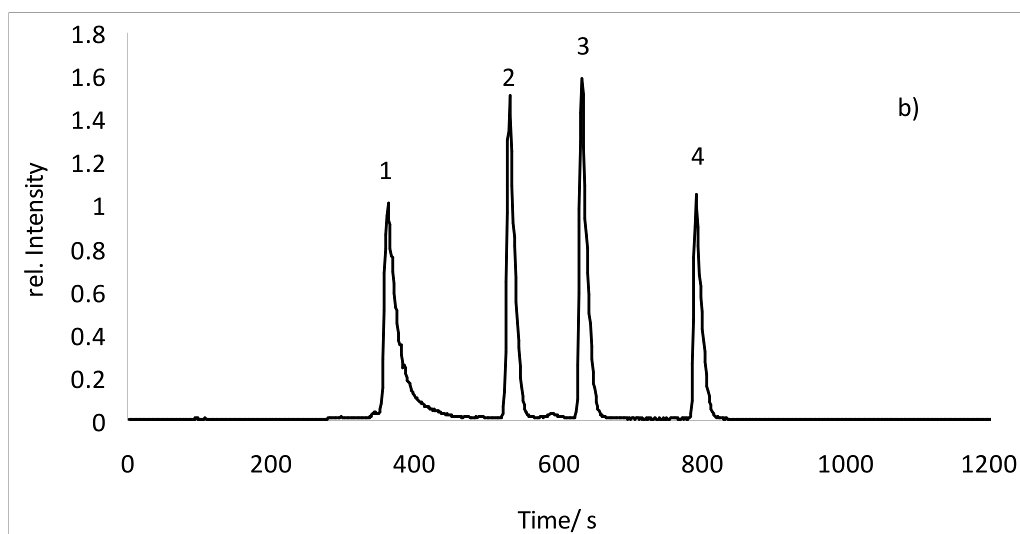
recoveries of only 5.2 % and 10.6 % respectively. The overall recovery was however around 90%, since the unstable species transformed to HO-Cbl, which almost tripled in concentration (280%). This confirms the recent study of Szterk et al.[9] who found that these transformations may be through oxidation in air and UV radiation, which result in the conversion of all physiologically important species to HO-Cbl. Hence, the samples need to be measured immediately after extraction.

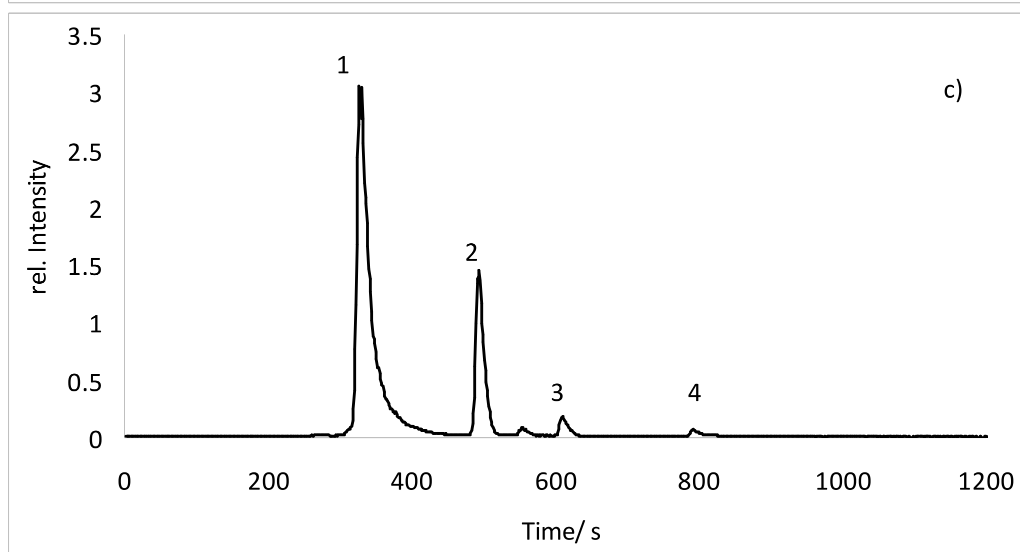
Stability of cobalamin species in different extractant solutions

To extract the different Cbl species from liver, they have to be liberated from their transport-proteins and transferred, unchanged, into the extract and the majority of the matrix should be removed. Several possibilities were tested for their influence on Cbl-speciation by spiking experiments using pig liver in order to address objective 1 and 2. Treatment of Cbl-standards with papain, diluted nitric acid, methanol or acetone resulted in species transformation. When all Cbl species were heated in acetone or methanol in order to precipitate all proteins all Cbl species eluted in the void and did not show the corrin-ring moiety (evident through missing $[M+H]^+$ data), this means transformation to unbound polar Co species took place (early eluting Co compounds close to the void volume). Hot water extraction at 50°C of the pig liver with subsequent measurement of Co speciation showed that part of Ado-Cbl in the pig liver was stable during this extraction (**Figure S2**). The majority of Co eluted however in the void (**Figure S3**). Spiking of all four Cbl species into the pig liver sample revealed also that the Me-Cbl and Ado-Cbl transferred mainly to HO-Cbl rather than unbound non-retarded Co. Hence, the reliable quantification of the two bioactive Cbl species was not possible. Since the aim is to have a sensitive method for total Cbl in contrast to any non-Cbl (inorganic Co), we tested the CN-Cbl method when all the Cbl species should be converted quantitatively to CN-Cbl (**Figure S4**). This method was originally developed for the extraction of Cbl species from serum (*2*). Quantitative conversion was tested by spiking pig liver with about 4 µg Co/g in form of all four Cbl species in triplicates, and study their stability in the KCN liver extracts.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60







272

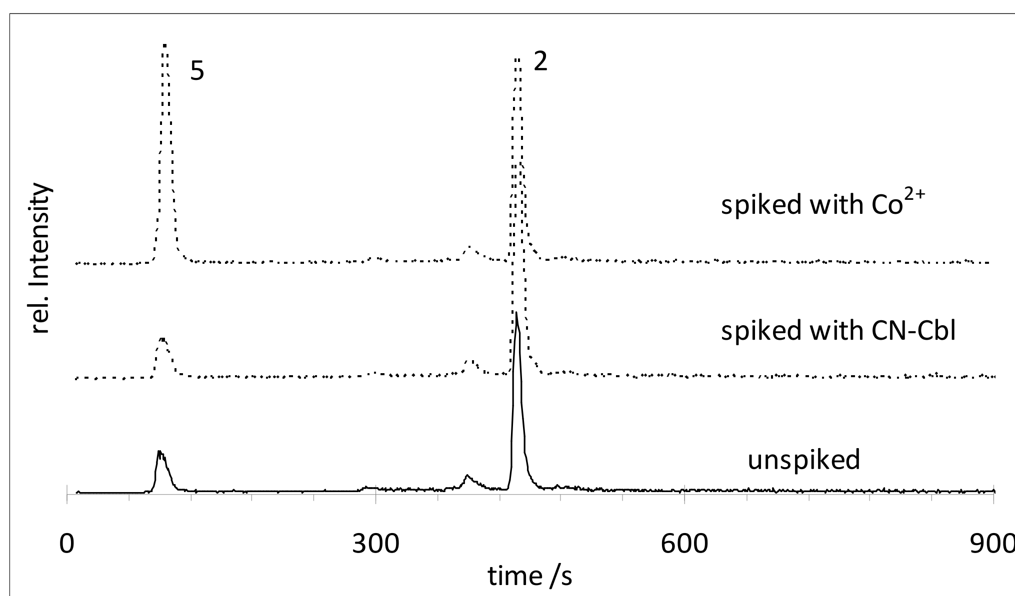
Figure 1a-c: Chromatographic separation of four different cobalamin species using reverse-phase HPLC detected on their $(M+H)^+$ and $(M+2H)^{2+}$ by ESI-Orbitrap MS (a) and simultaneously on m/z 59 for cobalt by ICP-MS (b) within 13 minutes. Peaks are 1) HO-Cbl (m/z 665, 1329.5), 2) CN-Cbl (m/z 678.5, 1355.5), 3) Ado-Cbl (m/z 790, 1580.5), 4) Me-Cbl (m/z 673, 1344.5). The degradation of a standard under oxygenated conditions at room temperature is shown in c).

To minimize the risk of underestimation of Cbl in liver samples Ado-Cbl and Me-Cbl were not determined as their individual species but rather quantitatively converted to CN-Cbl by the addition of sufficient cyanide. Additionally only one peak needs to be integrated which would make the SOP easier and lowers the error. When extracted with the aid of cyanide the resulting chromatogram shows only two Co peak, one for unbound early eluting Co and one for CN-Cbl as illustrated in **Figure 2**. None of the Cbl species seems to loose Co under the tested conditions. The spiked pig liver did not show an increase in the early eluting (unbound) Co, and only one prominent Co peak, that of CN-Cbl (**Figure 2**). The column recovery was around 95 %. The conversion of all spiked Cbl species to CN-Cbl was quantitative (94 +/- 2%; n=3) which renders this method to be accurate. Although the recovery of the spiked Cbl species was quantitative the extraction of Co_t was not (**Figure S5**). The Co_t concentration of the unspiked pig liver was 57 +/- 4.7 ng Co g^{-1} d.m. (**Figure S6**) while the Co_t determined in the extract was only 32 +/- 1.7 ng Co g^{-1} d.m., hence the extraction efficiency of Co_t was only 56 %. Since the nature of the unaccounted Co species was unknown the extraction method for Cbl was further optimized in order to prevent potential loss of Cbl species in the liver samples by varying the papain amount, the temperature and incubation time. The optimized extraction efficiency was 71 ± 28 % (n=4) of cobalt using between 10-50 mg liver 5 mg papain with 3 h incubation at 37°C. (**Figure S5**). Although the spiked Cbl gave an excellent precision of +/-2%, the precision of the intrinsic Co_t in the liver was higher (+/- 40 %) at the level of 4 µg Co as Cbl/g. This indicates that the liver samples were not homogeneous with regards to the Co_t when only 10-50 mg samples were used per digest. Hence, the homogeneity of the sample was investigated by using the XRF mapping (objective 3).

Using 3 times the standard deviation (SD) of the blank level, and a sample mass of 50 mg d.m., the method for has a detection limit of 0.18 ng/g d.m. for Co_t, while for the speciation for total Cbl a detection limit of 0.88 ng Co/g d.m. was established. A practical lower limit of quantification (10 times the SD of the blank) is therefore about

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

309 3 ng Co as Cbl/g d.m. liver. This means that the described analytical method is
310 capable to detect between 10-50 pg Co as Cbl (depending on the weight of the
311 sample). This is superior to all so far described methods.[2,9,12] This should be lower
312 than the expected levels of of Cbl in human fetal liver.
313
314
315

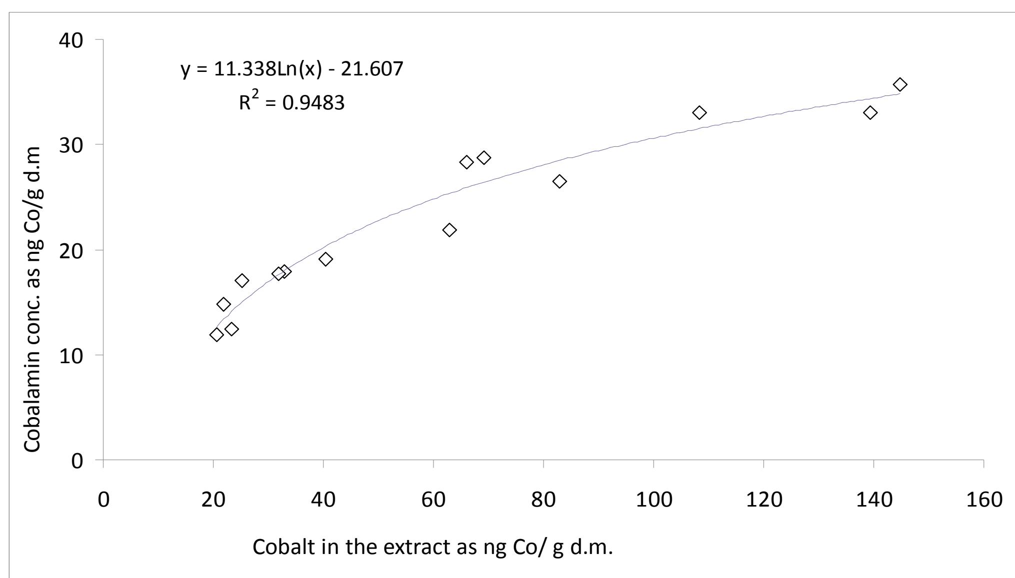


316
317 **Figure 2**: HPLC-ICP-MS chromatogram shows a pig liver extract using cyanide of an
318 unspiked and a CN-Cbl (peak 2) and Co^{2+} as nitrate spiked extracts (peak 5) gives the
319 inorganic cobalt in the extract, while peak 2 shows CN-Cbl.
320
321
322

323 Cobalt speciation in pig liver

324 The optimized method was applied to 14 pig liver samples. The amount of sample
325 used during this experiment was kept to below 50 mg per sample in order to evaluate
326 the suitability of the method for human fetal samples. Co_t varied significantly over
327 almost one order of magnitude (18-145 ng Co/g *d.m.*). The extraction efficiency was
328 measured for a subset (2 samples, n=3) and gave 89 ± 14 % with an outlier of only
329 43 % (**Figure S7**). All samples (n=14) were extracted for speciation analysis with the
330 optimized method. The extractable Co concentration ranged from 18 – 50 ng Co/g
331 d.m. Although the extraction efficiency was nearly quantitative, only a fraction of
332 total Co was in the form of Cbl measured as CN-Cbl (**Figure S8**). The Cbl fraction
333 accounted for 45 ± 15 % of Co_t, while non-specified unbound cobalt was nearly 55 %

1
2
3 334 with little unaccounted non-extractable Co. While Co_t concentration was highly
4
5 335 variable the Cbl-concentration was remarkable constant with 23 ± 8 ng Co/g *d.m*
6
7 336 (n=14). Interesting is the variation between the different liver samples; the liver
8 337 sample CC-5 contained around 68 % of Co as Cbl in the extracted material, while L1F
9
10 338 had only 25 %. This variability has been seen in beef livers before.[1] Considering only
11
12 339 the extractable Co, Cbl shows a logarithmic trend when related to extractable Co
13 340 (**Figure 3**). That indicates that high total Co concentrations in pig livers might not be
14
15 341 the result of vitamin B12 accumulation but rather of Co which is not bound as Cbl.
16
17 342 This contradicts that the amount of Vitamin B12 linearly depends on the amount of
18 343 Co_t in liver reported elsewhere.[1] Hence, the Cbl concentration cannot be estimated
19
20 344 from the total Co concentration in pig liver. The amount of Cbl needs to be measured
21
22 345 directly in order to give a reliable account of the vitamin B12 concentration.
23 346
24 347
25–42



43 348
44 349
45 350 **Figure 3:** Correlation cobalamin expressed as cobalt versus the cobalt concentration
46 351 in the extract of 14 pig liver samples.
47 352
48 353
49 354
50
51 355 In general, the results for the spiked pig liver suggest that full conversion of all Cbl
52
53 356 species to CN-Cbl was achieved. The separation of the Co species has been shown to
54 357 be robust (retention times did not vary more than 0.1 min) throughout the analysis.
55
56 358 Although pooled samples showed good reproducibility in their Co concentration
57
58 359 (approx. 5% **Figure S5**), subsamples taken from individual livers showed
59
60

considerable variability (**Figure S6 and S7**). This may suggest that cobalt is heterogeneously distributed throughout pig liver especially if only a small sample is taken, which would be unexpected for physiologically regulated Cbl.

To shed more light on the heterogeneity of Co and Cbl in the liver and whether Cbl species transformation had taken place during the sample preparation, i.e. the release of cobalt from the corrin ring, XRF mapping of the pig liver and subsequent μXANES was used for unspiked pig liver samples which showed qualitatively the occurrence of Ado-Cbl. The challenges to overcome were first the low concentration of cobalt < 0.1 mg/kg and the interference of the Fe Kβ fluorescence, which overlaps with Co Kα. Therefore Fe and Co were measured simultaneously and every pixel was corrected using Co Kα - Fe Kα/Kβ resulting in a cobalt specific map of the liver sample. The results clearly indicated the presence of Co in small hotspots (approximately 10 to 30 μm in size) throughout the samples (**Figure 4 and S9)**. The XANES spectra of the cobalt hotspots seem to be similar to inorganic Co^{+II} and Co^{+III} compounds and distinctively different from the XANES spectra of cobalamin standards characterized by a double feature in the main absorption peaks ($Co^{+II/III}$). Although the nature of these hotspots is unknown, it is not inconceivable that these hot spots are the result of absorbed cobalt containing particles. This would explain the heterogeneity of Co_t but the homogenous distribution of Cbl. Due to the above mentioned Fe interferences in these samples, a homogeneously low distribution of cobalamin in the sample would not be detected either by XRF or μXANES. However, XANES and XRF analysis suggests that the majority of Co in the pig livers was not in the form of Cbl but rather in the form on unbound Co^{+II}. Therefore, this confirmed the relatively low extraction efficiency of Co_t (70-80%) combined with the high recovery of spiked Cbl species. Hence, the described methodology with a low limit of detection (< 1 ng Co as Cbl/g d.m.) and its precision of < 5% and its accuracy of 94% it was suited to use for the determination of Cbl in fetal liver samples.

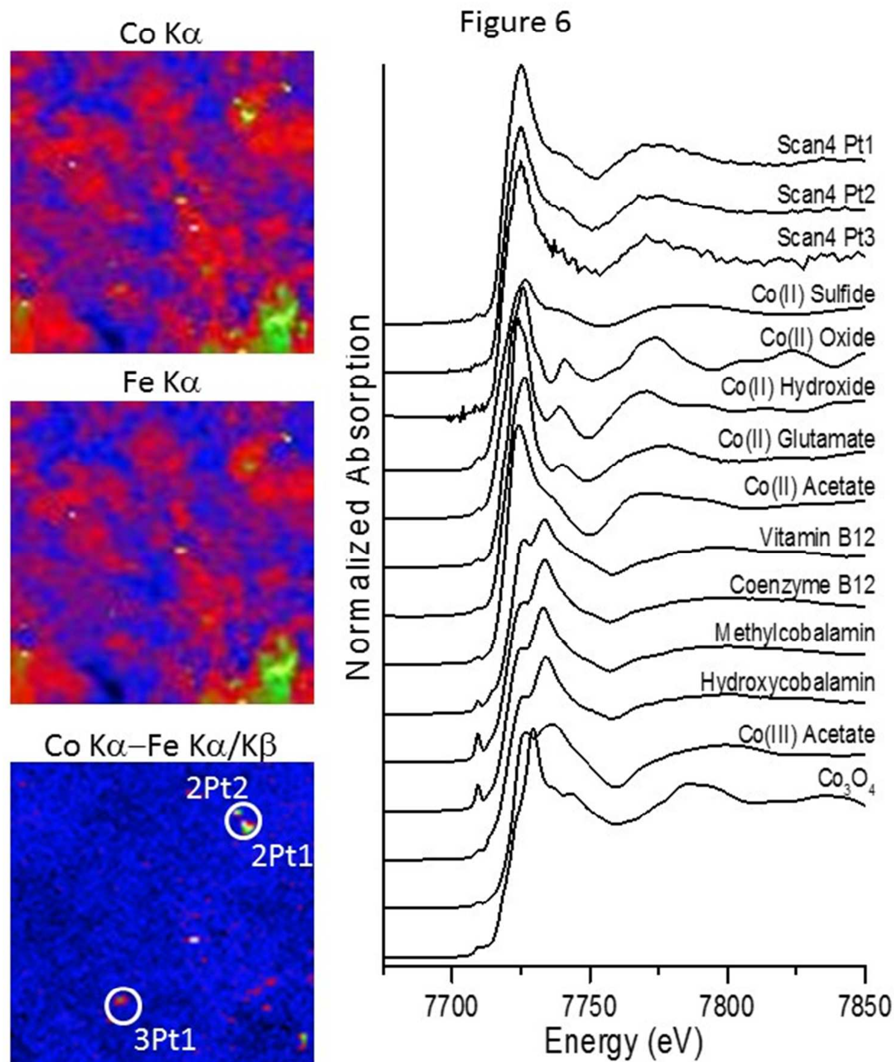


Figure 4a-b: 2 D cobalt map (1.5 x 1.5 mm)(a) from a shredded pig liver paste (resolution of about 20 µm with the µXANES spectra taken at the hotspots (b) in comparison to the XANES spectra of four different cobalamin standards.

Human fetal liver samples

Co_t in human fetal samples was analyzed in duplicate and showed a high variability (25 to 190 ng Co/g d.m., detection limit 2 ng/g *d.m*. **Figure 5**). All livers had Co_t and

ACS Paragon Plus Environment

1
2
3 398 Cbl concentrations above the detection limits. The first results of the study have been
4
5 399 published partly by Drake et al.[11] with regards to lifestyle influence on the Cbl
6
7 400 concentration in the fetal livers without describing the analytical method in detail.
8 401 Here, we describe the analytical method capable of measuring Cbl with high
9
10 402 sensitivity and precision and subsequent aspects of the study which enabled us to look
11
12 403 at a part of the study which was previously not described.
13 404 There was no correlation between fetal weight and the Co_t concentration neither was a
14
15 405 significant gender difference found (unpaired two-way ANOVA, p=0.082). The Co_t
16
17 406 concentrations were comparable with those reported in by Caldas and Dorea.[1] When,
18 407 however, the Cbl concentration was measured, a significant gender difference could
19
20 408 be established (two-way ANOVA, $p<0.05$). The Cbl concentration in the female liver
21
22 409 of non-smoking mothers was 643 ± 48 ng Vitamin B12/g dm, whereas male fetal liver
23 410 of non-smoking mothers contained 497 ± 51 ng Vitamin B12/g. The reason why there
24
25 411 is a gender difference is still unclear and how the C1 metabolism of the fetus is
26
27 412 influenced when the mother smokes has been discussed elsewhere.[11] The data also
28 413 indicate that the vitamin B12 concentration correlates linearly with the Co_t
29
30 414 concentration in the liver of the fetuses independent on the gender ($P<0.001$) (**Figure**
31
32 415 **5**).However, even if the correlation is significant the variability was still very large
33 416 within the data set and a precise measurement of Cbl needs to rely on direct
34
35 417 measurement rather than interpolation from total Co (**Figure 5**).
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

ACS Paragon Plus Environment

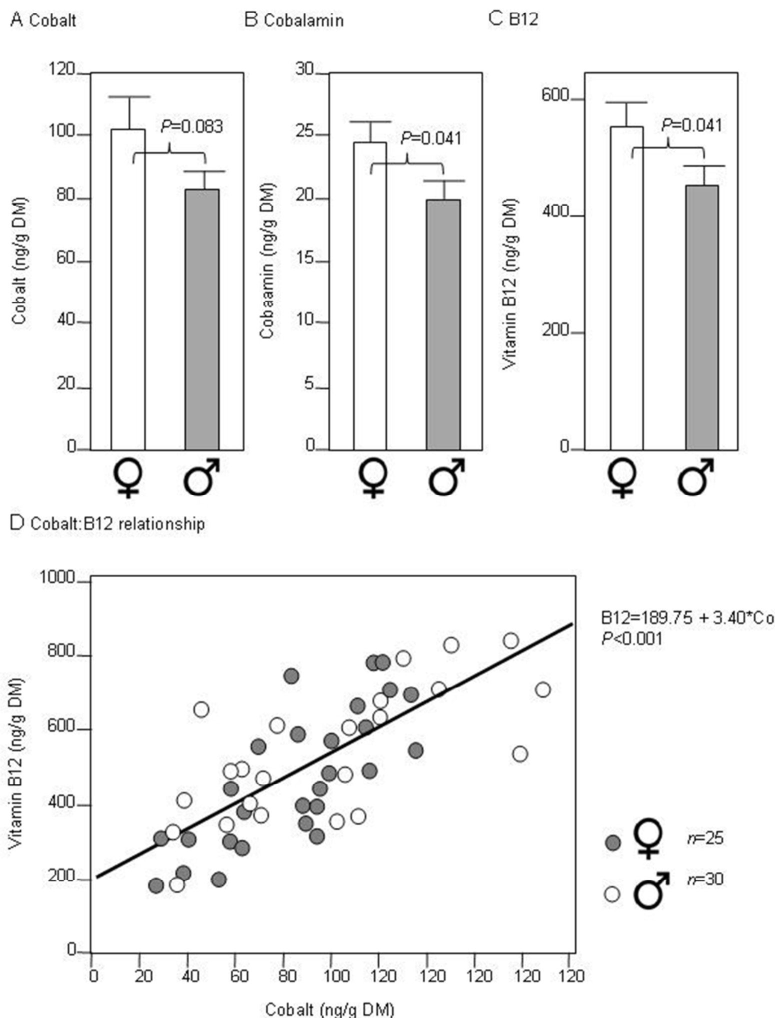


Figure 5: Co_t (A) and Cbl concentration (expressed as ng Co/g liver (B) and as ng Cbl/ g liver (C) in human fetal livers show a significant lower cobalamin level for female fetal livers. The p-values given are based on ANOVA unpaired two way tests. (D) shows the correlation of vitamin B12 and Co_t.

CONCLUSION

The method to determine cobalamin in liver samples described here is sensitive enough to determine background levels of unbound and bound cobalt in fetal liver samples. Although, we were unable to determine the individual physiologically active

forms of Cbl (Me-Abl and Ado-Cbl), all forms of Cbl could be transformed into CN-Cbl and determined quantitatively in liver samples with an accuracy of around 94 % and a precision of +/- 5 %. A significant amount of Co is present in a non-characterized form in the extract, which however is not an artefact of the extraction method or a degradation product of Cbl species. Not only is Co_t not representing the amount of Cbl in the liver samples, the analyte is subject to large variability through the accumulation of inorganic Co, which seem to point to particulate Co. The nature of this uncharacterized cobalt needs to be studied in the future.

ACKNOWLEDGEMENTS: S.G and J.B thank the Erasmus exchange program of the EU. Although EPA contributed to this article, the research presented was not performed by or funded by EPA and was not subject to EPA's quality system requirements. Consequently, the views, interpretations, and conclusions expressed in this article are solely those of the authors and do not necessarily reflect or represent EPA's views or policies. Sector 20 facilities at the Advanced Photon Source, and research at these facilities, are supported by the US Department of Energy - Basic Energy Sciences, the Canadian Light Source and its funding partners, and the Advanced Photon Source. Use of the Advanced Photon Source, an Office of Science User Facility operated for the U.S. Department of Energy (DOE) Office of Science by Argonne National Laboratory, was supported by the U.S. DOE under Contract No. DE-AC02-06CH11357.

We thank Ms Margaret Fraser and Ms Samantha Flannigan for their expert assistance. The staff at Grampian NHS Pregnancy Counselling Service were essential for collecting fetuses.

Funding: Chief Scientist Office (Scottish Executive, CZG/1/109 (P.A.F.), & CZG/4/742 (P.A.F.); NHS Grampian Endowments 08/02 (P.A.F.); the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement no 212885 (P.A.F.); the Medical Research Council grants MR/L010011/1 (P.A.F.).

References

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

[1] Caldas, E.D.; Dorea, J.G. *J. Nutri. Biochem.* **1992**, *3*, 539-542.

[2] Kumar, S.S.; Chouhan, R.S.; Thakur, M.S. *Anal. Biochem.* **2010,** *398,* 139-149.

[3] Carmel, R. *Am. J. Clin. Nutr.* **2011**, doi: 10.3945/ajcn.111.013441.

[4] Lindenbaum, J.; Rosenberg, I.H.; Wilson, P.W.F.; Stabler, S.P.; Allen, R.H. *Am. J. Clin. Nutri.* **1994**, *60,* 2-11.

5 Chassaigne, H.; Łobiński, R. *Anal. Chim. Acta* **1998**, 227-235.

[6] Kelly, R.J.; Gruner, T.M.; Sykes, A.R. *Biomed. Chromatogr.* **2005**, *19,* 329-333.

[7] Jndar Koyyalamudi, R.A.O.; Jeong, S.C.; Yip Cho, K.A.I.; Pang, G. *J. Agri. Food Chem*. **2009**, *57*, 6327-6333.

[8] Lipiec, E.; Ruzik, L.; Zhou, Y.; Jarosz, M.; Polec-Pawlak, K. *J. Anal. At. Spectrom.* **2011,** *26*, 608-612.

[9]Szterk, A.; Roszko, M.; Malek, K.; Czerwonka, M.; Waszkiewicz-Robak, B. *Meat Sci*. **2012**, *91,* 408-413.

[10] Feldmann, J.; Salaun, P.; Lombi, E. *Environ. Chem.* **2009**, *6*, 275-279.

[11] Drake, A.J.; O'Shaughnessy, P.J.; Aonteiro, A.; Kerrigan, D.; Goetz, S.; Raab, A.; Rhind, S.M.; Sinclair, K.D.; Meharg, A.A.; Feldmann, J.; Fowler, P.A. *BMC Medicine* **2015**, *13,* Art. Nr. 18

[12] Rappazzo, M.E.; Salmi, H.E.; Hall, C.A. *Brit. J. Haematol.* **1970**, *18*, 425-433.

[13] Bluemlein, K.; Raab, A.; Feldmann, J. *Anal. Bioanal. Chem.* **2009**, *393*, 357-366.

[14] Ravel, B.; Newville, M. *J. Synchrot. Radiat*. **2005,** *12*, 537-541.

[15] Amayo, K.O.; Petursdottir, A.; Newcombe, C.; Gunnlaugsdottir, H.; Raab, A,; Krupp, E.M.; Feldmann, J. *Anal. Chem.* **2011**, *83*, 3589-3595.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60