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**PAPER**

# Absolute quantification of transferrin in blood samples of harbour seals using HPLC-ICP-MS†

**Mechthild Grebe,*[ab] Daniel Pröfrock,*[a] Antje Kakuschke,[a] Jose A. C. Broekaert[b] and Andreas Prange[a]**

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Harbour seals (*Phoca vitulina*) are bio-indicators for the assessment of their habitat and environmental changes. Besides population parameters and trends (survival, age structure, sex ratio), the individual health status represents a further important parameter for this assessment. The health status of seals is a complex and vague term, determined by a wide range of diagnostic parameters. Quantities of important blood proteins such as transferrin (Tf), as well as altered distribution patterns of its glycoforms, are frequently used as biomarkers in clinical diagnosis. Within this context Tf quantities and a varying pattern of its glycoforms are used as indicator for *e.g.* certain liver diseases, which also represents one of the most frequently observed pathological indication in harbour seals of the North Sea. Currently, most assay based quantification methods for Tf are limited since they often provide only information regarding the total Tf concentration rather than information of its different glycoforms. Due to a lack of suitable seal Tf antibodies also the application of more specific antibody based approaches is not possible. Within this background a new approach for the absolute quantification of the iron-transport protein Tf in the blood of harbour seals using its characteristic iron content and HPLC-ICP-MS detection is described. Method validation was performed using a certified human serum reference material (ERM-DA470K/IFCC). A Tf concentration of 2.33 ± 0.03 g $L^{-1}$ (sum of all quantified glycoforms) has been calculated, which is in good agreement with the certified total Tf concentration of 2.35 ± 0.08 g $L^{-1}$, confirming the accuracy of the proposed analytical method. Finally, different seal samples were analysed to demonstrate the suitability of the procedure for the quantification of Tf in real samples as well as to observe modified glycoform patterns. Compared to our previous studies for the first time it was possible to quantify the serum Tf baseline reference range for male (1.42–2.35 g $L^{-1}$) and female German North Sea seals (1.93–2.74 g $L^{-1}$) as well as a CDT level of 0.00–0.10 g $L^{-1}$, respectively, which provides valuable further diagnostic information regarding the health status of these specific marine mammals. Compared to assay based quantification approaches the proposed technique indicates great potential to obtain comparable and traceable absolute quantitative results, which are in particular important for long term investigations. This absolute quantification is based on an accurate, traceable element standard, while assay based approaches often show variations depending on the kit quality or changing activities of the used antibodies.

## Introduction

As top predators within the marine food web marine mammals such as harbour seals (*Phoca vitulina*) can be utilised as indicators for medium and long term changes of their natural habitat. Besides population parameters and trends (survival, age structure, sex ratio), the individual health status is an important parameter for this assessment. Their health status is in particular impaired by several mainly anthropogenic influences, *e.g.* shipping, fishery, contaminants and operation of offshore wind parks and can be determined by a wide range of diagnostic parameters. The accurate determination of contamination levels of different environmental compartments, such as sediments or the water column, besides measuring body burdens of selected organisms such as harbour seals[1–12] represents an established strategy for ecosystem studies or

[a] *Helmholtz-Zentrum Geesthacht, Institute for Coastal Research, Marine Bioanalytical Chemistry, Max-Planck-Str. 1, 21502 Geesthacht, Germany. E-mail: mechthild.grebe@hzg.de, daniel.proefrock@hzg.de*
[b] *University of Hamburg, Department of Chemistry, Institute for Inorganic and Applied Chemistry, Martin-Luther-King-Platz 6, 20146 Hamburg, Germany*

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environmental assessment. Several studies revealed the correlation between bioaccumulation of environmental contaminants in the tissues of marine mammals and suppression effects on their immune system, which in consequence increases their vulnerability against pathogens or infectious diseases.[13–15] To obtain more complete diagnostic information a wide range of parameters is necessary, such as immunological investigations and biochemical analysis of enzymes or selected marker proteins.[16] Within this context the iron transporting glycoprotein transferrin (Tf) represents a further possible marker protein for the description of the health status of harbour seals, whose quantity as well as altered distribution patterns of its glycoforms are used as biomarker in human medicine *e.g.* for several liver diseases,[17–19] which also represent one of the most frequently observed pathological indication in harbour seals of the North Sea.[20]

The lack of standardised, accurate quantification methods represents a general problem for the application of relevant biomarkers in environmental analysis as well as their continuous monitoring. Currently, assay based methods dominate the analysis of diagnostically relevant biomarkers such as Tf. Turbidimetric immunoassays[21] or combined approaches, which include isoelectric focusing, immunoblotting and laser densiometry,[18,22] are commonly used for the quantification of Tf. Unfortunately, these techniques often provide only information of the total Tf concentration rather than its different diagnostically relevant glycoforms. Due to the lack of suitable antibodies such methods are further restricted to selected species, such as humans or important domestic animals. First studies with respect to Tf and its glycoforms as well as its potential as bio-marker in seals has been shown by Grebe *et al.* and Kakuschke *et al.*[9,23] New possibilities for its specific quantitative determination arise in particular from the current developments in proteomics and bio inorganic speciation analysis respectively.[24] The chromatographic separation of Tf glycoforms relies on their individual isoelectric point (*pI*), which is determined by the sialic acid as well as the iron content of every single glycoform. To eliminate the influence of a varying iron content on the separation, most HPLC based Tf assays include an iron saturation step to provide an uniform iron load of the Tf molecule, whose two theoretical binding sites for $Fe^{3+}$ are normally only partly occupied. For human serotransferrin of healthy individuals the saturation is about 30%.[25,26] The known iron stoichiometry as well as the high stability constant of the Tf-Fe complex (ranging from $10^{-19}$–$10^{-22}$ $M^{-1}$ for human Tf as specified in the literature[27–30]) facilitates the application of sensitive element specific detection methods, such as inductively coupled plasma mass spectrometry (ICP-MS) hyphenated to HPLC for the separation and detection of this protein.[23,31] Del Castillo Busto demonstrates the application of iron isotope dilution analysis (IDA) for the quantification of human carbohydrate deficient transferrin (CDT), which is considered as a reliable marker for chronic alcohol abuse.[32] IDA can provide highly accurate quantitative results; however, it has to be kept in mind that it requires expensive, isotopically enriched element spikes as well as different additional time consuming daily measurements when aiming at highest analytical accuracy. This includes spike characterisation and quantification, mass bias corrections and mass flow determination of the spike, as well as numerous calculations to finally transfer the measured isotope ratios into mass flow chromatograms, which makes this method only partly suitable for routine analysis with high sample numbers.

The application of a compound independent calibration strategy using simple certified as well as traceable element standards represents a further approach for the accurate Tf quantification *via* its iron content, which is feasible due to the compound independent elemental response of ICP-MS.[33] However, also this strategy is challenging, in particular when working with gradient elution, as required for the HPLC separation of the different Tf glycoforms. As described in the literature, the matrix composition of an LC eluent strongly influences the ionisation properties of an ICP. The constant introduction of a defined amount of carbon containing matrix compounds (*e.g.* organic solvents)[34,35] or certain gases (*e.g.* nitrogen, helium)[36,37] to the ICP can lead to an improved ionisation process for difficult to ionise elements such as As, Se or P. This may result in improved sensitivities, while contrary effects such as ionisation suppression are frequently observed, when exceeding the optimal level, *e.g.* when switching from isocratic to gradient conditions or when introducing other matrix such as salts gradients into the plasma. To minimize this limitation some reports describe the utilisation of mathematical correction functions,[38–40] the application of membrane desolvation to remove *e.g.* organic solvents from the sample aerosol[41,42] or the application of *constant post column sheath flows* to buffer solvent related changes in the elemental response during gradient elution.[43–45] To overcome the problems related with the application of a constant sheath flow for buffering gradient related effects during gradient LC separation Pröfrock *et al.*,developed an analytical approach, using a matched *reversed gradient post column sheath flow*. This approach maintains the percentage of organic solvent, which finally enters the plasma at a constant level over the whole gradient. In addition an automated flow injection analysis (FIA) at the end of each gradient separation has been implemented, which allows the injection of a certified element calibration standard for the absolute quantification of the separated compounds.[46] Within this background the aim of this study was to adapt the last mentioned approach as an alternative method for the fast, straightforward, reliable absolute quantification of Tf glycoforms in serum samples of a rarely investigated but environmentally relevant animal species such as harbour seals. The quantitative determination of Tf as well as its glycoforms provides valuable diagnostic information regarding the health status of the investigated animals, which may support the future assessment of their environment.

## Experimental

### Chemicals, standards and material

Ultrapure water (18 MΩ cm) was prepared using a Millipore Elix 3/Milli-Q element water purification system (Millipore, Milford, MA, USA). Single element standards of Fe, Co, Ge and Cs, as well as $Na_2CO_3$, $FeCl_3$, acetic acid and ammonia

solution were obtained from Merck at the highest available quality (Merck KGaA, Darmstadt, Germany). $MgCl_2$, Dextransulfate and Bis-Tris buffer were purchased from Fluka (Fluka, Buchs, Switzerland). A 10fold stock solution of the Bis-Tris buffer was cleaned by passing the solution through Chelex® 100 resin (Fluka) before its dilution to the required concentration level.

Argon 5.0 (99.999% purity) was used as plasma gas. Hydrogen 5.0 (99.999% purity) was used as cell gas inside the octopole reaction system during all experiments. Both gases were obtained from Air Liquide (Air Liquide, Lübeck, Germany). Solutions, standards and samples were prepared under clean room conditions (class 1000) inside a clean bench (class 100) to avoid contamination. To reduce possible sample degradation, all handling and preparation steps of the plasma and serum samples were performed under cooled conditions (4 °C) if not otherwise stated.

## Instrumentation

### HPLC

An Agilent 1100 series liquid chromatography system consisting of two four channel on-line degassers, two binary LC pump, a cooled autosampler, a column oven, a diode array UV detector (DAD) equipped with a standard flow cell as well as a cooled fraction collector were used during all experiments. All connections were made of 1/16″ o.d., 125 μm i.d. PEEK tubings and zero dead volume fittings (Upchurch Scientific/GAT Analysen Technik, Bremen, Germany). For reducing the iron background a passivation procedure using nitric acid recommended by the producer was applied to the system.

Flow injection analysis has been performed using a six port inert electronic switching valve (Rheodyne, Model LABPro, Cotati, CA, USA) equipped with a home made injection loop made out of PEEKsil tubing.

All LC modules were arranged with respect to achieve the lowest possible dead volume between the LC setup and the ICP-MS. A splitter has been used to allow parallel UV detection (at 280 and 460 nm respectively) as well as on-line fraction collection of the separated and quantified Tf species to conduct further experiments. The gradient conditions for pump 2 producing the reversed gradient are delayed for compensating the column dead volume. The time-shift of pump 2 is 0.5 min over the whole gradient. Details of the chromatographic conditions as well as further instrumental settings are given in Tables 1 and 2.

### ICP-MS

For element specific detection and quantification of the separated Tf glycoforms an Agilent 7500cs ICP-MS system (Agilent Technologies, Tokyo, Japan) was used as detector. It features an on-axis octopole ion guide operated in an RF-only mode, which is used as collision/reaction cell with respect to reduce the abundance of interfering polyatomic ions. To reduce the background, especially on the main isotope $^{56}Fe$, which is highly interfered by polyatomic ions such as $^{40}Ar^{16}O^+$, $H_2$ with an optimum flow rate of 5 mL $min^{-1}$ was used during all experiments. In addition kinetic energy discrimination obtained by the settings of the octopole and quadrupole bias was applied to further reduce the background on the main iron isotope. A detailed description regarding the principles of kinetic energy discrimination can be found elsewhere.[47–49]

A micro concentric nebuliser (PFA 100, Elemental Scientific, Omaha, Nebraska, USA) combined with the standard quartz double pass Scott spray chamber supplied with the ICP-MS instrumentation was used for the hyphenation of the HPLC system to the ICP-MS. Details of the ICP-MS settings are also given in Table 1.

**Table 1** Instrumental conditions

| | |
|---|---|
| **HPLC Agilent 1100** | |
| SAX Column | PorosHQ, 2.1 mm × 100 mm, 10 μm particles |
| Mobile Phase | A: 20 mM Bis-Tris, pH 6.5, 10 μg $L^{-1}$ Ge<br>B: 20 mM Bis-Tris, 500 mM Ammonium acetate, pH 6.5, 10 μg $L^{-1}$ Ge, Cs |
| Injection volume | 50 μL |
| Flow rate | 0.75 mL $min^{-1}$ |
| Column oven | 30 °C |
| **ICP-MS Agilent 7500cs** | |
| RF power | 1600 W |
| Carrier gas | 0.92 L $min^{-1}$ |
| Makeup gas | 0.20 L $min^{-1}$ |
| Extraction lens 1 | 5 V |
| Extraction lens 2 | −180 V |
| Octopole bias | −18 V |
| Quadrupole bias | −16 V |
| Cell gas | 5 mL $min^{-1}$ $H_2$ |
| Spray chamber Temperature | 4 °C |
| Measured isotopes | $^{54}Fe$, $^{56}Fe$, $^{57}Fe$, $^{59}Co$, $^{72}Ge$, $^{133}Cs$ |
| Dwell time | 0.1 s |

**Table 2** Summary of the gradient settings for the separation and quantification of different Tf glycoforms

| Time [min] | Pump 1 [%B] | Pump 2 [%B] | Time [min] | Contact external valve |
|---|---|---|---|---|
| (a) Settings for human serum samples (ERM®-A470k/IFCC) | | | | |
| 0 | 0 | 30 | | |
| 2 | 0 | 30 | | |
| 22 | 30 | 0 | 21 | closed = inject |
| | | | 22 | open = load |
| | | | 24 | closed = inject |
| | | | 25 | open = load |
| | | | 27 | closed = inject |
| 28 | 30 | 0 | 28 | open = load |
| 30 | 100 | 0 | | |
| 35 | 100 | 0 | | |
| 40 | 0 | 30 | | |
| 45 | 0 | 30 | | |
| (b) Settings for seal serum samples | | | | |
| 0 | 0 | 12 | | |
| 2 | 0 | 12 | | |
| 26 | 12 | 0 | 26 | closed = inject |
| | | | 27 | open = load |
| | | | 29 | closed = inject |
| | | | 30 | open = load |
| | | | 32 | closed = inject |
| 33 | 12 | 0 | 33 | open = load |
| 35 | 100 | 0 | | |
| 41 | 100 | 0 | | |
| 45 | 0 | 12 | | |
| 50 | 0 | 12 | | |

## Instrumental setup for gradient compensation and flow injection analysis based calibration and absolute protein quantification

The instrumental setup used during all experiments is shown in Fig. 1. For the separation of the Tf glycoforms with an anion exchange column and the detection of the Tf-Fe complex by ICP-MS a second HPLC pump was used to generate a counter current gradient sheath flow, which is comparable with the setup described by Pröfrock and Prange.[46] The counter current gradient sheath flow was mixed after the separation column with the eluent coming from the column outlet, using a mixing tee. For compensating the dead volume of the column the reversed gradient generated by the second pump was adjusted with a 0.5 min delay. In consequence during the separation a constant mobile phase composition enters the nebuliser and finally the plasma, which results in constant plasma conditions and therefore a constant elemental response of the ICP-MS. This allows the utilisation of a one point calibration *via* flow injection of an iron element standard solution (Merck KGaA) to calibrate the whole chromatogram. The flow injection was generated by a sample loop connected to an electronically controlled external six port valve, operated by the Agilent ChemStation software *via* an additional four channel contact board installed inside the autosampler.

## Samples

The serum samples have been obtained during five seal catch campaigns carried out on the Düne Helgoland (H) and on the Lorenzenplate (L), German North Sea in 2006 and 2007. The seal catch campaigns were conducted within the seal management plan of the Trilateral Monitoring and Assessment Program (TMAP) between Denmark, Germany and the Netherlands. The health monitoring includes the sampling of blood from wildlife harbour seals. The samplings were carried out under the administration of the Research and Technology Centre (FTZ) in Büsum, Germany according to the relevant laws and institutional guidelines. All experiments were approved by the Schleswig-Holstein Wadden Sea National Park Office and the Ministry of Agriculture, the Environment and Rural Areas of Schleswig-Holstein. The caught animals were restrained manually to assess length, weight, sex and age as well as to collect anal smears and blood samples. During the procedure the animals were continuously under the observation of a veterinarian. After completing the investigations, the animals were released back into the wildlife.

Blood was collected into Serum Gel S monovettes (Sarstedt AG & Co, Nümbrecht, Germany) after puncture of the epidural vertebral vein using a 20 mL syringe and a 12 mm $\times$ 100 mm needle (TSK-Supra, TSK Laboratory, Japan). The tubes were kept at room temperature until further sample processing. The blood samples were processed within 1 to 12 h. Serum was separated by centrifugation and aliquots were frozen until further use.

For this study, samples of 43 animals were selected (see Table 4). The nonparametric Mann-Whitney U-Test was used for comparison of Tf and CDT concentrations for male and female animals, for the two age groups and for both locations; significance is indicated at $P < 0.05$.

For method validation and as control sample a certified reference material of human serum (ERM®-DA470k/IFCC; European Commission, JRC, IRMM, Geel, Belgium) was used during all experiments.

### Sample preparation

All sample handling and manipulation were carried out under cooled conditions (4 °C) inside a class 100 clean bench, with

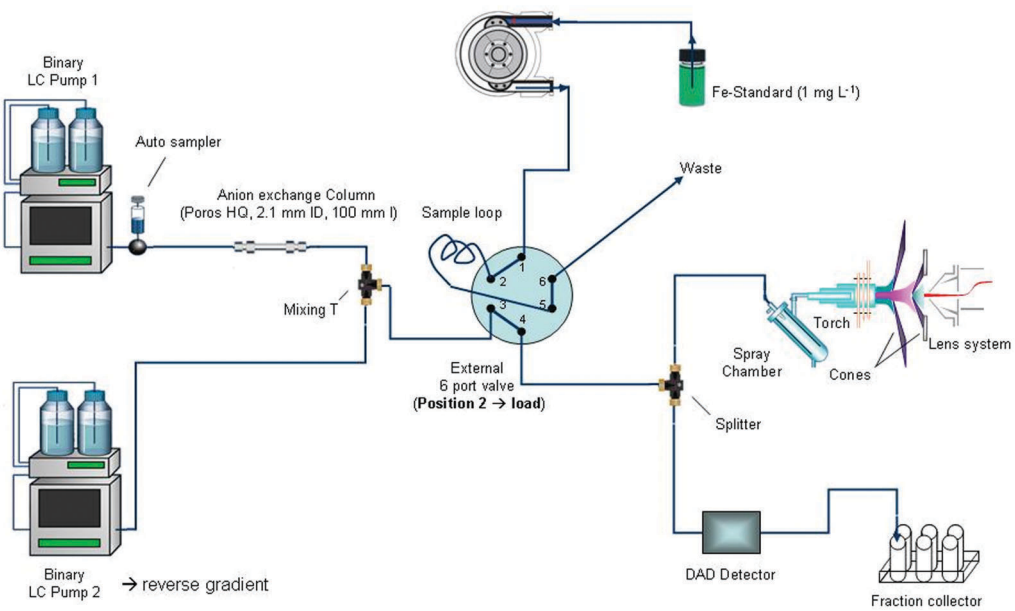


**Fig. 1** Schematic drawing of the instrumental setup for the straightforward absolute quantification of Tf glycoforms using HPLC-ICP-MS and a compound independent FIA calibration

the aim to minimise contaminations and sample degradation, respectively.

Since the absolute quantification requires a uniform iron stoichiometry of the different Tf glycoforms, an iron saturation procedure was developed based on a method described by del Castillo Busto *et al.*[31,32] To improve the long term stability of the chromatographic column as well as to remove additional interfering matrix components from the serum samples, the original method was further optimised by the implementation of a lipoprotein precipitation step similar to the one described by Jeppsson *et al.*[50] The description of this combined sample preparation procedure can be found in the ESI.† Details also can be found in Grebe *et al.*[23]

## Results and discussion

In the following sections the fast and targeted quantification of Tf as well as its glycoforms, whose quantity and pattern may provide diagnostically relevant information, will be demonstrated using HPLC hyphenated to collision/reaction cell ICP-MS as separation and detection method.

### Investigation of gradient related effects on the iron specific detection

The highly resolution separation of Tf glycoforms *via* anion exchange chromatography depends on the application of a suitable salt gradient. A gradient of a 20 mM Bis-Tris buffer at pH 6.5 containing 500 mM ammonium acetate has been found to be sufficient for the separation when using a PorosHQ column. Table 2 summarises the gradient conditions optimised for seal serum samples as well as those optimised for the separation of a certified human Tf reference material, which was used for further method validation. To investigate possible effects on the iron specific detection caused by the increasing matrix load during the gradient elution, which is necessary for the separation of the individual Tf glycoforms, repetitive flow injection analysis with a simple inorganic iron standard (8.7 ng absolute) was performed, while applying a salt gradient. As shown in Fig. 2a a strong effect on the iron detection was observed, as indicated by the dots inside the figure reflecting the average peak area calculated after three repetitive measurements. The measured peak areas continuously drop proportional with the increasing salt intake into the plasma. The salt gradient was monitored by the $^{133}$Cs spike, which has been added exclusively to solvent B at a concentration of 10 μg $L^{-1}$. Even though only up to 12% of the salt containing mobile phase B were introduced into the plasma at the end of the gradient separation, a high peak area RSD of 8.94% was calculated, which hampers an accurate quantitative analysis. In consequence, this results in a loss of 20.4% of the average peak area when comparing the last flow injection at a concentration of 60 mM of ammonium acetate with the average peak area of the first iron flow injection, which is not effected by the salt gradient (0 mM of ammonium acetate). Further details on the gradient conditions are listed in Table 2.

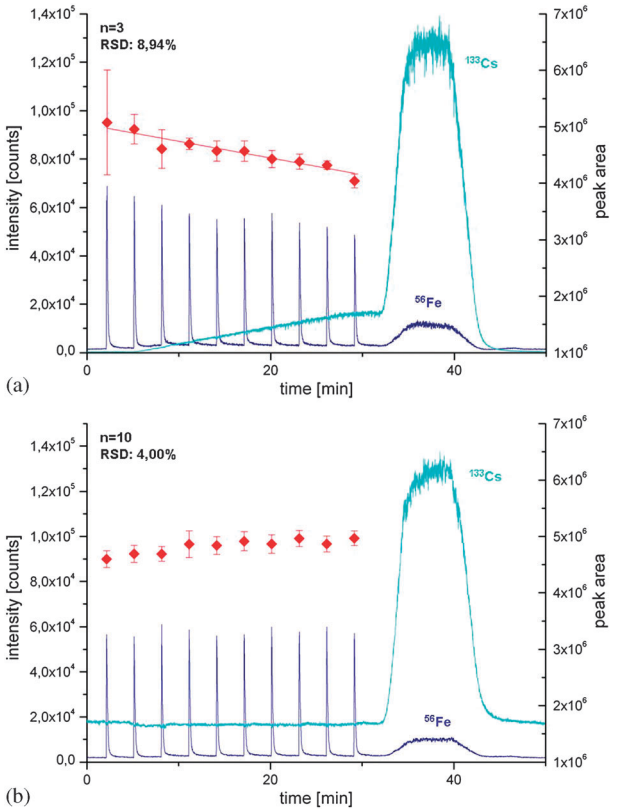


**Fig. 2** (a) Effect of the ammonium acetate gradient on the iron specific detection during repetitive flow injection of an iron standard (8.7 ng). The dots indicate the average peak area ($n$ = 3. RSD 8.94%) calculated from three individual experiments measured using the $^{56}$Fe isotope. (b) Application of the reversed gradient sheath flow approach to compensate salt gradient related effects on the iron specific detection. The dots again indicate the average peak area ($n$ = 10. RSD 4.00%) calculated from three individual experiments measured using the $^{56}$Fe isotope.

### Compensation of salt gradient related effects on the iron specific detection

To overcome this detrimental effects a second HPLC pump was used to generate a matched reversed gradient sheath flow. Again a gradient of a 20 mM Bis-Tris buffer at pH 6.5 containing 500 mM ammonium acetate was used, while applying repetitive flow injections with a simple inorganic iron standard. This time a matched reversed gradient sheath flow was mixed at a post column location with the eluent coming from the separation column, which allows the application of gradient conditions for the separation, while the element specific detection was performed under isocratic conditions (30 mM of ammonium acetate) due to the equilibrating effect of the reversed gradient sheath flow. As shown in Fig. 2b, the influence of the gradient on the iron specific detection (compare also with Fig. 2a) could be minimised, as demonstrated by the constant peak areas, indicated by the dots inside the figure, which reflect the average peak area calculated after 10 repetitive measurements at specific time points. Only a small peak area RSD of 4% was calculated, which is mandatory for accurate quantitative analysis. The stability of the $^{133}$Cs signal also indicates the precise interplay of both pump systems, which is

a prerequisite for the accurate equilibration of the gradient. The reduced detection capability for low concentrated sialoforms due to the dilution effect caused by the reversed gradient as well as the additional coasts of the necessary second LC pump represents the only disadvantage of the proposed instrumental setup. Initial experiments using capillary LC with respect to reduce this dilution problem have been already conducted. Such setups based on total consumption nebulisers show a nearly 100% transport efficiency into the plasma, which partly compensates the dilution effect.

### Determination of the sample loop volume

The Tf quantification is based on flow injection experiments at the end of every chromatographic separation to calibrate the iron specific detection. Initial experiments indicate that the specified volume of the previously used, commercially available PEEK sample loops was of limited precision, which results in inaccurate quantitative results. However, to allow an accurate absolute quantification of the separated Tf glycoforms *via* their characteristic iron content the precision of the FIA calibration step is of great importance. In order to exactly specify the injected sample volume during FIA a home made sample loop consisting of a PEEKsil capillary with an i.d. of 200 μm ± 5 μm and a length of 200 mm was used. To calculate the volume of the sample loop as well as to account for additional dead volumes generated by the fittings and the rotor/strator unit, a $^{59}$Co standard solution with a concentration of 1 g $L^{-1}$ was used, as described recently by Giusti *et al.*[43] After connecting the specified sample loop to the external six port valve, the standard was continuously pumped through the sample loop using the internal peristaltic pump of the ICP-MS. Afterwards the content of the sample loop was injected by switching the valve position into a carrier stream of 3% $HNO_3$ at a flow rate of 0.5 mL $min^{-1}$. A volume of about 1 mL was collected 19 times at the valve outlet and precisely weighted using a micro balance. Finally the $^{59}$Co concentration in solution was measured by ICP-MS against an external calibration, which allows the calculation of the total injection volume of the specified setup. A theoretical volume of 6.283 μL ± 0.318 μL was calculated for the used PEEKsil capillary based on the precisely measured tube length and the inner diameter specified by the manufacturer. In contrast the used setup indicates an effective injection volume of 8.722 μL ± 0.074 μL, which corresponds to a volume deviation of about 5% compared to the theoretical value. This is mainly a result of the additional dead volume caused by the fittings and the cavities on the surface of the internal rotor of the used six port valve. The measured volume was used for all further calculations.

### Investigation of the saturation of seal Tf with iron

The accurate quantification of Tf and its glycoforms *via* its specific iron content relies on a constant iron stoichiometry of the Tf molecule. To evaluate the optimal iron concentration for the improved iron saturation procedure, which is necessary to ensure the complete saturation and therefore uniform iron stoichiometry of every Tf molecule, the effect of different iron concentrations on the saturation procedure were tested. Therefore, aliquots of the same seal serum sample were used for the saturation procedure, while adding different amounts of iron to the reaction mixture. Afterwards, the samples were processed as described in the experimental section and finally measured using HPLC-ICP-MS to quantify the iron content of the main Tf glycoform. Fig. S-1a shows the saturation curve of Tf from a seal serum sample. As demonstrated in Fig. S-1a, a constant Tf-Fe level was obtained at an iron concentration in the final reaction mixture exceeding 15 mg $L^{-1}$. At higher iron concentrations no further improvements in the reaction yield were obtained. To keep the remaining iron content, which is injected with the sample onto the column as low as possible, all saturation reactions were conducted with a final iron concentration in the reaction mixture of at least 40 mg $L^{-1}$, which is equal to a 6 fold excess, based on a Tf concentration of 2.8 g $L^{-1}$.

In order to allow complete iron saturation also the necessary reaction times were evaluated. Therefore, a partly saturated Tf sample was analysed by HPLC-ICP-MS, while aliquots of the same sample were processed using the iron saturation procedure. In Fig. S-1b it is demonstrated, that the iron saturation of Tf represents a fast process since already after 8 min no further enhancement of the measured peaks areas, even after extended incubation times, were observed. In consequence, all further experiments were performed using a total incubation time of 60 min.

### Analytical figures of merit

**Detection limit, limit of quantification.** Under the given instrumental conditions iron detection limits of 170 pg (S/N $\geq$ 3) and a limit of quantification of 440 pg (S/N $\geq$ 10) have been calculated, which corresponds to approximately 123 μg and 320 μg Tf, respectively. The detection limit was in particular hampered by the relatively high iron background caused by impurities of the used Bis-Tris buffer chemicals even though they were declared as ''high purity grade''. Currently a depletion procedure is under development, which utilizes Chelex 100 resin to reduce the iron as well as other trace element contaminations of the final buffer solution.

**Evaluation of the method accuracy.** Since no reference material is available for seal Tf as well as for its glycoforms a certified human serum reference material (ERM®-DA470k/IFCC) was used for the validation of the proposed analytical method. This includes the iron saturation step, a lipoprotein precipitation and the final FIA based calibration/quantification after using anion exchange HPLC-ICP-MS. Fig. 3 shows a typical chromatogram of the reference material using the main $^{56}$Fe isotope for the element specific determination of the different Tf glycoforms present in the reference material. After the separation the individual glycoforms were quantified based on their known iron content. A Tf concentration of 2.33 ± 0.03 g $L^{-1}$ (sum of all quantified glycoforms) was calculated, which is in good agreement with the certified total Tf concentration of 2.35 ± 0.08 g $L^{-1}$ of the reference material, confirming the method accuracy.

**Repeatability of the chromatographic separation.** When using the reversed gradient approach, reproducible separations with retention times and Tf concentration RSDs below 0.5% and

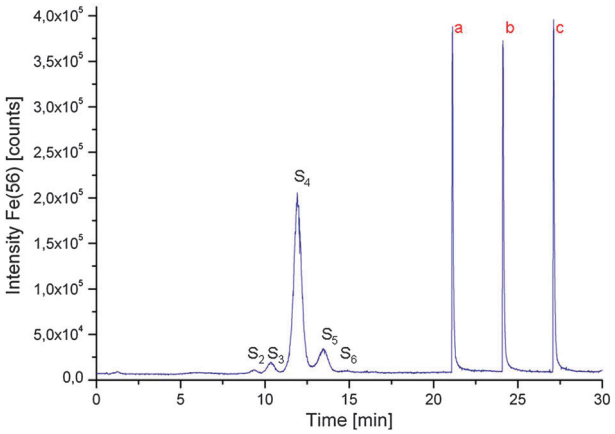


**Fig. 3** A typical chromatogram obtained during the analysis of the certified reference material (ERM®-DA470k/IFCC) using the main $^{56}Fe$ isotope for the element specific determination of the different Tf glycoforms present in the reference material.

5.6%, respectively, were obtained for the separation of the certified reference serum (ERM®-DA470k/IFCC). This also demonstrates the precise interplay of the two pump systems, which is a prerequisite for accurate gradient equilibration. Detailed results for the Tf glycoforms are also summarised in Table S-1, ESI.†

### Quantification of seal Tf and its glycoforms

Using the optimised chromatographic conditions it was possible to separate up to nine different glycoforms, depending on the seal sample used in less than 30 min.[23]

In comparison to IDA based approaches the calibration step adds additional 5–6 min to each separation since a prolonged waiting time in between the three FIA experiments has been choosen to allow the Fe signal to reach the baseline level again. Here further optimisation may be possible to achieve separation times comparable to those of IDA setups, however, even 5 min more a negliable keeping in mind the

**Table 3** Tf and CDT concentration with standard deviation (SD) and the relative CDT level (CDT/Tf*100) for serum samples of 43 harbour seals (*Phoca vitulina*) of the North Sea from the location Helgoland (H) and Lorenzenplate (L), juvenile (≤2 years) and adult (>2 years) seals, male (m) and female (f) animals

| Seal | Location | Age (year) | Sex | Tf (g $L^{-1}$) | SD (g $L^{-1}$) | CDT (g $L^{-1}$) | SD (g $L^{-1}$) | CDT/Tf*100 (%) |
|---|---|---|---|---|---|---|---|---|
| **1** | L | >2 | **m** | **1.62** | 0.01 | **0.00** | 0.00 | **0.0** |
| **2** | L | >2 | **m** | **1.56** | 0.04 | **0.00** | 0.00 | **0.0** |
| **3** | L | >2 | **m** | **1.98** | 0.03 | **0.04** | 0.03 | **2.0** |
| **7** | L | >2 | **m** | **2.16** | 0.05 | **0.10** | 0.01 | **4.6** |
| **8** | L | >2 | **m** | **1.97** | 0.14 | **0.00** | 0.00 | **0.0** |
| **9** | L | ≤2 | **m** | **1.76** | 0.07 | **0.00** | 0.00 | **0.0** |
| **10** | L | >2 | **m** | **2.19** | 0.13 | **0.10** | 0.04 | **4.6** |
| **11** | L | >2 | **m** | **2.06** | 0.10 | **0.02** | 0.00 | **1.0** |
| **12** | L | >2 | **m** | **2.36** | 0.09 | **0.06** | 0.06 | **2.7** |
| **14** | L | ≤2 | **m** | **1.30** | 0.03 | **0.04** | 0.01 | **3.4** |
| **15** | L | ≤2 | **m** | **1.48** | 0.12 | **0.02** | 0.01 | **1.6** |
| **16** | L | >2 | **m** | **2.16** | 0.01 | **0.02** | 0.00 | **0.9** |
| **17** | L | ≤2 | **m** | **2.29** | 0.08 | **0.01** | 0.00 | **0.6** |
| **19** | L | ≤2 | **m** | **2.14** | 0.04 | **0.08** | 0.01 | **3.6** |
| **20** | L | ≤2 | **m** | **1.75** | 0.09 | 0.21 | 0.08 | 12.0 |
| **21** | L | ≤2 | **m** | **2.15** | 0.11 | **0.08** | 0.02 | **3.7** |
| **23** | L | >2 | **m** | **1.93** | 0.09 | **0.07** | 0.00 | **3.6** |
| **25** | L | >2 | **m** | **2.27** | 0.05 | **0.07** | 0.01 | **3.1** |
| **26** | L | >2 | **m** | **1.92** | 0.09 | **0.06** | 0.01 | **3.1** |
| **27** | L | >2 | **m** | **2.24** | 0.04 | 0.51 | 0.05 | 22.8 |
| **28** | L | >2 | **m** | **1.77** | 0.04 | **0.05** | 0.01 | **2.8** |
| **29** | L | >2 | **m** | **2.27** | 0.10 | **0.02** | 0.00 | **0.9** |
| **30** | L | >2 | **m** | **2.45** | 0.07 | 0.25 | 0.02 | 10.2 |
| **32** | L | >2 | **m** | **2.27** | 0.10 | **0.10** | 0.01 | **4.4** |
| **33** | L | >2 | **m** | **2.35** | 0.03 | **0.09** | 0.01 | **3.8** |
| **34** | L | >2 | **m** | **1.64** | 0.10 | **0.07** | 0.02 | **4.3** |
| **35** | L | >2 | **m** | **1.77** | 0.10 | **0.09** | 0.02 | **5.1** |
| **36** | L | >2 | **m** | **1.93** | 0.10 | **0.10** | 0.01 | **5.2** |
| **37** | H | ≤2 | **m** | **1.26** | 0.09 | **0.05** | 0.03 | **4.0** |
| **38** | H | >2 | **m** | **2.27** | 0.08 | **0.13** | 0.01 | **5.7** |
| **39** | H | >2 | **m** | **2.02** | 0.07 | **0.06** | 0.01 | **3.0** |
| **41** | H | >2 | **m** | **1.83** | 0.08 | **0.05** | 0.01 | **2.7** |
| **42** | H | >2 | **m** | **1.77** | 0.06 | **0.03** | 0.01 | **1.7** |
| **43** | H | >2 | **m** | **1.92** | 0.04 | **0.09** | 0.00 | **4.7** |
| **4** | H | ≤2 | **f** | **2.63** | 0.11 | **0.03** | 0.01 | **1.1** |
| **5** | H | ≤2 | **f** | **2.05** | 0.07 | 0.19 | 0.03 | 9.3 |
| **6** | H | >2 | **f** | **2.45** | 0.08 | **0.03** | 0.01 | **1.2** |
| **13** | L | ≤2 | **f** | **2.18** | 0.11 | **0.00** | 0.00 | **0.0** |
| **18** | L | >2 | **f** | **2.22** | 0.25 | **0.02** | 0.00 | **1.0** |
| **22** | L | ≤2 | **f** | **2.54** | 0.06 | **0.08** | 0.01 | **3.1** |
| **24** | L | >2 | **f** | **1.85** | 0.06 | 0.33 | 0.06 | 17.8 |
| **31** | L | >2 | **f** | **2.04** | 0.11 | **0.06** | 0.03 | **2.9** |
| **40** | H | ≤2 | **f** | **2.81** | 0.07 | 0.89 | 0.05 | 31.7 |

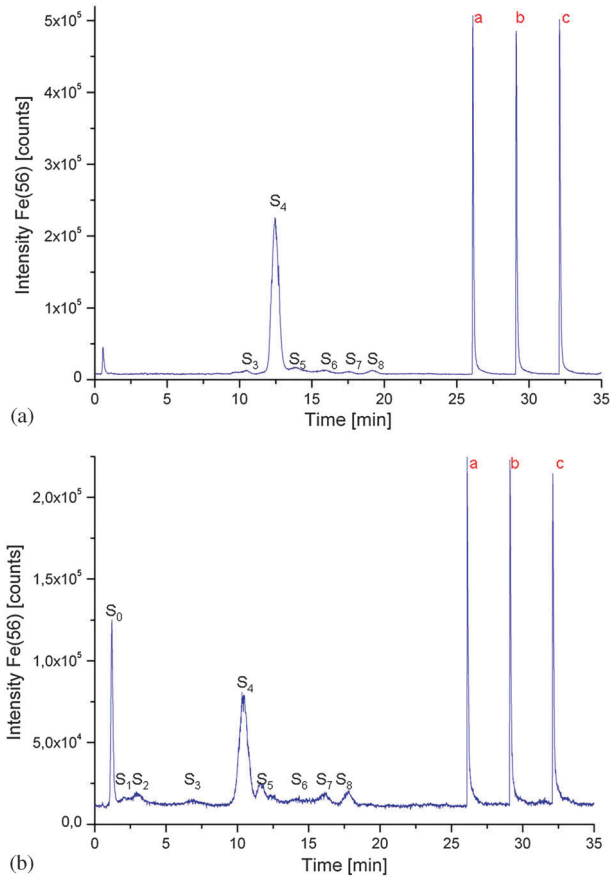


**Fig. 4** (a) A typical chromatogram of a seal serum sample (seal 3) measuring the main $^{56}Fe$ isotope for the element specific determination of the different Tf glycoforms. (b) A chromatogram of a seal serum sample with elevated CDT value (seal 40) measuring the main $^{56}Fe$ isotope for the element specific detection.

total required analysis time of about 45 min for one sample (including column washing and equilibration).

For the first time, the absolute quantification of Tf and its glycoforms was performed in samples of 43 harbour seals of the German North Sea. The total Tf concentration and also the CDT concentration of this seal serum samples are listed in Table 3 and as example, one typical chromatogram is shown in Fig. 4a. The normal seal Tf glycoform pattern is similar to the pattern known from human Tf (Fig. 3), the high abundant main glycoform $S_4$ is surrounded by different low abundant glycoforms. In general the lower sialinated glycoforms show shorter retention times compared to the $S_4$ form due to their less negative charge, while the higher sialinated glycoforms generally elute later in comparison to the main $S_4$ form. The lower sialinated glycoforms, with none, one or two sialic acid residues are abnormal variations. The sum of a-, mono- and disialo-Tf glycoforms are defined as the CDT concentration.[31,32]

A minimal Tf concentration of 1.26 g $L^{-1}$ and a maximal concentration of 2.81 g $L^{-1}$ in seal samples were measured (see Tables 3 and 4), while a median of 2.05 g $L^{-1}$ Tf in serum of the 43 harbour seals was calculated. No significant differences between the two locations and age groups were observed. Between male and female animals the differences were marginally significant ($P = 0.013$). For further studies a baseline reference range (5–95% percentile) of 1.42–2.35 g $L^{-1}$ Tf can be estimated for males. The range (5–95% percentile) for females was slightly higher with 1.93–2.74 g $L^{-1}$ Tf in serum (Table 5).

Significant differences of the CDT level for this sample set of 43 harbour seals, between location, age and sex were not found. However, the glycoform pattern of six animals (seal 5, seal 20, seal 24, seal 27, seal 30 and seal 40) was different from the normal pattern shown in Fig. 4a. In all six cases the CDT glycoforms showed elevated concentrations, as example one chromatogram of a seal serum sample with an elevated CDT value (seal 40) is shown in Fig. 4b. These glycoform patterns bear similarities to the patterns shown by Kakuschke *et al.* 2010[9] for W02/08Pv and W04/08Pv. The maximum concentration of the elevated CDT in serum measured is 0.89 g $L^{-1}$, which is related to the measured overall Tf concentration corresponding to a CDT level of 31.7%.

The baseline reference range for the CDT concentration was calculated of the remaining 37 animals with a normal Tf glycoform pattern without any pathologic findings. A baseline reference range (5–95% percentile) of 0.00–0.10 g $L^{-1}$ CDT can be estimated, which corresponds to a CDT level of 0.0–5.1% (Table 4).

Currently, the elevated CDT values could not be correlated with a set of other clinical parameter and therefore, further investigations are necessary also with respect to extend the dataset. In particular, more data for female animal are needed. This is mainly caused due to the fact that surprisingly more male than female animals have been caught during the different campaigns.

**Table 4** Statistic parameter for Tf and CDT concentration of serum samples of the 43 harbour seals (*Phoca vitulina*)

| | Male Tf (g $L^{-1}$) | Female Tf (g $L^{-1}$) | Combined Tf (g $L^{-1}$) | Normal glycoform pattern | | Elevated CDT level | |
|---|---|---|---|---|---|---|---|
| | | | | CDT (g $L^{-1}$) | CDT/Tf*100 (%) | CDT (g $L^{-1}$) | CDT/Tf*100 (%) |
| **Median** | 1.97 | 2.22 | **2.05** | 0.05 | 2.9 | 0.29 | 14.9 |
| **Average** | 1.97 | 2.31 | 2.04 | 0.05 | 2.6 | 0.40 | 17.3 |
| **STDEV** | 0.31 | 0.32 | 0.34 | 0.04 | 1.7 | 0.27 | 8.7 |
| **Min** | 1.26 | 1.85 | **1.26** | 0.00 | 0.0 | 0.19 | 9.3 |
| **Max** | 2.45 | 2.81 | **2.81** | 0.13 | 5.7 | **0.89** | **31. 7** |
| **Number** | 34 | 9 | 43 | 37 | 37 | 6 | 6 |
| **Percentile 5%** | **1.42** | **1.93** | 1.49 | **0.00** | **0.0** | 0.19 | 9.5 |
| **Percentile 95%** | **2.35** | **2.74** | 2.53 | **0.10** | **5.1** | 0.80 | 29.5 |

## Conclusion

The proposed HPLC-ICP-MS method illustrates the possibilities, that arise due to the skilful application of elemental mass spectrometry for clinical or environmental related applications, such as the absolute quantification of proteins with a high diagnostic relevance such as transferrin.

For the first time, to our knowledge, Tf and its glycoforms were quantified for a set of serum samples of wildlife harbour seals from the German North Sea. A median of 2.05 g $L^{-1}$ Tf in serum was estimated. The concentrations in serum of female animals were marginally significant higher than in males. The normal CDT level for male and female as well as for juvenile and adult seals from both locations was below 6%, for the glycoform patterns without important pathological findings. For further studies a baseline reference range of 1.42–2.35 g $L^{-1}$ Tf for males, of 1.93–2.74 g $L^{-1}$ Tf for females and of 0.00–0.10 g $L^{-1}$ CDT can be used.

In general, this quantification approach can be easily adapted also to the absolute quantification of other relevant metalloproteins, which contain ICP-MS detectable element tags at a known, stable stoichiometry. In comparison to IDA based strategies for the quantification of metalloproteins, whose applicability is restricted to those elements, which show at least two stable isotopes, also proteins, which contain monoisotopic element tags can be quantified by this generic approach. The proposed approach requires additional infrastructure (a second LC pump) however due to its simplicity, this method is in particular interesting for the routine analysis of Tf glycoforms *e.g.* as conducted in clinical routine labs. In comparison to enzymatic or immuno assay based approaches for the quantification of Tf, the application of HPLC-ICP-MS is in general advantageous because it can be utilised for every species since this approach does not depend on the availability of specific antibodies. Therefore, it can be also easily applied to the analysis of uncommon animal species such as harbour seals. In addition it provides glycoform specific information, which can not be obtained with most other methods.

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## References

1 V. N. de Jonge, M. Elliott and V. S. Brauer, Marine monitoring: Its shortcomings and mismatch with the eu water framework directive's objectives, *Mar. Pollut. Bull.*, 2006, **53**(1–4), 5–19.
2 A. T. Fisk, C. A. de Wit, M. Wayland, Z. Z. Kuzyk, N. Burgess, R. Letcher, B. Braune, R. Norstrom, S. Polischuk Blum, C. Sandau, E. Lie, H. J. S. Larsen, J. U. Skaare and D. C. G. Muir, An assessment of the toxicological significance of anthropogenic contaminants in canadian arctic wildlife, *Sci. Total Environ.*, 2005, (351–352), 57–93.
3 R. J. Law, P. Bersuder, L. K. Mead and P. D. Jepson, Pfos and pfoa in the livers of harbour porpoises (phocoena phocoena) stranded or bycaught around the uk, *Mar. Pollut. Bull.*, 2008, **56**(4), 792–797.
4 J. Mi, A. Orbea, N. Syme and M. Ahmed, Peroxisomal proteomics, a new tool for risk assesment of peroxisome proliferating in the marine environment, *Proteomics*, 2005, **5**(15), 3954–3965.
5 S. Mössner and K. Ballschmitter, Marine mammals as global pollution indicators for organochlorines, *Chemosphere*, 1997, **34**(5–7), 1285–1296.
6 S. Fahrenholtz, S. Griesel, D. Pröfrock and A. Kakuschke, Essential and non-essential elements in tissues of harbour porpoises (phocoena phocoena) stranded on the coasts of the north and baltic seas between 2004–2006, *J. Environ. Monit.*, 2009, **11**(5), 1107–1113.
7 S. Griesel, A. Kakuschke, U. Siebert and A. Prange, Trace element concentrations in blood of harbor seals (phoca vitulina) from the wadden sea, *Sci. Total Environ.*, 2008, **392**(2–3), 313–323.
8 S. Griesel, R. Mundry, A. Kakuschke, S. Fonfara, U. Siebert and A. Prange, Mineral elements and essential trace elements in blood of seals of the north sea measured by total-reflection X-ray fluorescence analysis, *Spectrochim. Acta, Part B*, 2006, **61**, 1158–1165.
9 A. Kakuschke, E. Valentine-Thon, S. Griesel, J. Gandrass, O. Pérez Luzardo, L. Domínguez Boada, M. Zumbado Peña, M. Almeida González, M. Grebe, D. Pröfrock, H.-B. Erbsloeh, K. Kramer, S. Fonfara and A. Prange, First health and pollution study on harbor seals (phoca vitulina) living in the german elbe estuary, *Mar. Pollut. Bull.*, 2010, **60**(11), 2079–2086.
10 A. Kakuschke, S. Griesel, S. Fonfara, T. Rosenberger and A. Prange, Concentrations of selected essential and non-essential elements in blood of harbor seal (phoca vitulina) pups of the german north sea, *Biol. Trace Elem. Res.*, 2009, **127**(1), 28–36.
11 A. Kakuschke and A. Prange, The influence of metal pollution on the immune system—a potential stressor for marine mammals in the north sea, *International Journal of Comparative Psychology*, 2007, **20**(2), 179–193.
12 A. Kakuschke, E. Valentine-Thon, S. Griesel, S. Fonfara, U. Siebert and A. Prange, The immunological impact of metals in harbor seals (phoca vitulina) of the north sea, *Environ. Sci. Technol.*, 2005, **39**(19), 7568–7575.
13 P. M. Bennett, P. D. Jepson, R. J. Law, B. R. Jones, T. Kuiken, J. R. Baker, E. Rogan and J. K. Kirkwood, Exposure to heavy metals and infectious disease mortality in harbour porpoises from england and wales, *Environ. Pollut.*, 2001, **112**(1), 33–40.
14 P. D. Jepson, P. M. Bennett, C. R. Allchin, R. J. Law, T. Kuiken, J. R. Baker, E. Rogan and J. K. Kirkwood, Investigating potential associations between chronic exposure to polychlorinated biphenyls and infectious disease mortality in harbour porpoises from england and wales, *Sci. Total Environ.*, 1999, **244**, 339–348.
15 U. Siebert, C. Joiris, L. Holsbeek, H. Benkes, K. Failing, K. Frese and E. Petzinger, Potential relation between mercury concentrations and necropsy findings in cetaceans from german waters of the north and baltic seas, *Mar. Pollut. Bull.*, 1999, **38**(4), 285–295.
16 M. P. Cajaraville, M. J. Bebianno, J. Blasco, C. Porte, C. Sarasquete and A. Viarengo, The use of biomarkers to assess the impact of pollution in coastal environments of the iberian peninsula: A practical approach, *Sci. Total Environ.*, 2000, **247**(2–3), 295–311.
17 T. Arndt, Carbohydrate-deficient transferrin as a marker of chronic alcohol abuse: A critical review of preanalysis, analysis, and interpretation, *Clinical Chemistry*, 2001, **47**(1), 13–27.
18 A. Helander, G. Eriksson, H. Stibler and J.-O. Jeppsson, Interference of transferrin isoform types with carbohydrate-deficient transferrin quantification in the identification of alcohol abuse, *Clinical Chemistry*, 2001, **47**(7), 1225–1233.
19 Y. Murawaki, H. Sugisaki, I. Yuasa and H. Kawasaki, Serum carbohydrate-deficient transferrin in patients with nonalcoholic liver disease and with hepatocellular carcinoma, *Clin. Chim. Acta*, 1997, **259**(1–2), 97–108.
20 U. Siebert, P. Wohlsein, K. Lehnert and W. Baumgärtner, Pathological findings in harbour seals (phoca vitulina): 1996–2005, *J. Comp. Pathol.*, 2007, **137**(1), 47–58.
21 A. Helander, M. Fors and B. Zakrisson, Study of axis-shield new%cdt immunoassay for quantification of carbohydrate-deficient transferrin (cdt) in serum, *Alcohol and Alcoholism*, 2001, **36**(5), 406–412.

 

22 R. Anton and P. Bean, 2 methods for measuring carbohydrate-deficient transferrin in inpatient alcoholics and healthy controls compared, *Clinical Chemistry*, 1994, **40**(3), 364–368.
23 M. Grebe, D. Pröfrock, A. Kakuschke, J. A. C. Broekaert and A. Prange, Metallomics approach for the identification of the iron transport protein transferrin in the blood of harbour seals (phoca vitulina), *Metallomics*, 2010, **2**(10), 683–693.
24 J. A. Hagger, M. B. Jones, D. R. Leonard, R. Owen and T. S. Galloway, Biomarkers and integrated enviormental risk assessment: Are there more questions than answers?, *Integr. Environ. Assess. Manage.*, 2006, **2**(4), 312–329.
25 E. del Castillo Busto, M. Montes-Bayon, J. I. Garcia Alonso, J. A. Caruso and A. Sanz-Medel, Novel hplc-icp-ms strategy for the determination of β2-transferrin, the biomarker of cerebrospinal fluid (csf) leakage, *Analyst*, 2010, **135**(7), 1538–1540.
26 A. Van Campenhout, C. Van Campenhout, A. R. Lagrou and B. Manuel-y-Keenoy, Effects of *in vitro* glycation on $Fe^{3+}$ binding and $Fe^{3+}$ isoforms of transferrin, *Clin. Chem.*, 2004, **50**(9), 1640–1649.
27 S. L. Byrne, A. N. Steere, N. D. Chasteen and A. B. Mason, Identification of a kinetically significant anion binding (kisab) site in the n-lobe of human serum transferrin, *Biochemistry*, 2010, **49**(19), 4200–4207.
28 R. R. Crichton, S. Wilmet, R. Legssyer and R. J. Ward, Molecular and cellular mechanisms of iron homeostasis and toxicity in mammalian cells, *J. Inorg. Biochem.*, 2002, **91**(1), 9–18.
29 J.-M. El Hage Chahine and R. Pakdaman, Transferrin, a mechanism for iron release, *Eur. J. Biochem.*, 1995, **230**(3), 1102–1110.
30 W. Kaim and B. Schwederski, *Bioanorganische chemie zur funktion chemischer elemente in lebensprozessen*, 3. überarbeitet Auflage ed. Teuber studienbücher chemie, ed. C. Elschenbroich, F. Hensel and H. Hopf, überarbeitete Auflage, B. G. Teuber Verlag/GWV Fachverlag GmbH, Stuttgart, 2004, vol. 3.
31 M. E. del Castillo Busto, M. Montes-Bayon, E. Blanco-Gonzalez, J. Meija and A. Sanz-Medel, Strategies to study human serum transferrin isoforms using integrated liquid chromatography icpms, maldi-tof, and esi-q-tof detection: Application to chronic alcohol abuse, *Anal. Chem.*, 2005, **77**(17), 5615–5621.
32 M. E. del Castillo Busto, M. Montes-Bayon and A. Sanz-Medel, Accurate determination of human serum transferrin isoforms: Exploring metal-specific isotope dilution analysis as a quantitative proteomic tool, *Anal. Chem.*, 2006, **78**(24), 8218–8226.
33 E. Svantesson, J. Pettersson and K. E. Markides, The use of inorganic elemental standards in the quantification of proteins and biomolecular compounds by inductively coupled plasma spectrometry, *J. Anal. At. Spectrom.*, 2002, **17**(5), 491–496.
34 F. R. AbouShakra, M. P. Rayman, N. I. Ward, V. Hotton and G. Bastian, Enzymatic digestion for the determination of trace elements in blood serum by inductively coupled plasma mass spectrometry, *J. Anal. At. Spectrom.*, 1997, **12**(4), 429–433.
35 E. H. Larsen and M. B. Ludwigsen, Determination of iodine in food-related certified reference materials using wet ashing and detection by inductively coupled plasma mass spectrometry, *J. Anal. At. Spectrom.*, 1997, **12**(4), 435–439.
36 S. F. Durrant, Alternatives to all-argon plasmas in inductively-coupled plasma-mass spectrometry (icp-ms)—an overview, *Fresenius' J. Anal. Chem.*, 1993, **347**(10–11), 389–392.
37 N. N. Sesi, A. Mackenzie, K. E. Shanks, P. Y. Yang and G. M. Hieftje, Fundamental-studies of mixed-gas inductively-coupled plasmas, *Spectrochim. Acta, Part B*, 1994, **49**(12–14), 1259–1282.
38 C. Siethoff, I. Feldmann, N. Jakubowski and M. Linscheid, Quantitative determination of DNA adducts using liquid chromatography electrospray ionization mass spectrometry and liquid chromatography high-resolution inductively coupled plasma mass spectrometry, *J. Mass Spectrom.*, 1999, **34**(4), 421–426.
39 M. Wind, H. Wesch and W. D. Lehmann, Protein phosphorylation degree: Determination by capillary liquid chromatography and inductively coupled plasma mass spectrometry, *Anal. Chem.*, 2001, **73**(13), 3006–3010.
40 N. Zinn, B. Hahn, R. d. Pipkorn, D. Schwarzer and W. D. Lehmann, Phosphorus-based absolutely quantified standard peptides for quantitative proteomics, *J. Proteome Res.*, 2009, **8**(10), 4870–4875.
41 M. Edler, N. Jakubowski and M. Linscheid, Styrene oxide DNA adducts: Quantitative determination using p-31 monitoring, *Anal. Bioanal. Chem.*, 2005, **381**(1), 205–211.
42 M. Edler, N. Jakubowski and M. Linscheid, Quantitative determination of melphalan DNA adducts using hplc—inductively coupled mass spectrometry, *J. Mass Spectrom.*, 2006, **41**(4), 507–516.
43 P. Giusti, D. Schaumlöffel, J. R. Encinar and J. Szpunar, Interfacing reversed-phase nanohplc with icp-ms and on-line isotope dilution analysis for the accurate quantification of selenium-containing peptides in protein tryptic digests, *J. Anal. At. Spectrom.*, 2005, **20**(10), 1101–1107.
44 A. Pereira Navaza, J. R. Encinar, M. Carrascal, J. Abian and A. Sanz-Medel, Absolute and site-specific quantification of protein phosphorylation using integrated elemental and molecular mass spectrometry: Its potential to assess phosphopeptide enrichment procedures, *Anal. Chem.*, 2008, **80**(5), 1777–1787.
45 A. Pereira Navaza, J. R. Encinar and A. Sanz-Medel, Absolute and accurate quantification of protein phosphorylation by using an elemental phosphorus standard and element mass spectrometry, *Angew. Chem., Int. Ed.*, 2007, **46**(4), 569–571.
46 D. Pröfrock and A. Prange, Compensation of gradient related effects when using capillary liquid chromatography and inductively coupled plasma mass spectrometry for the absolute quantification of phosphorylated peptides, *J. Chromatogr., A*, 2009, **1216**(39), 6706–6715.
47 P. Leonhard, R. Pepelnik, A. Prange, N. Yamada and T. Yamada, Analysis of diluted sea-water at the ng l-1 level using an icp-ms with an octopole reaction cell, *J. Anal. At. Spectrom.*, 2002, **17**(3), 189–196.
48 S. D. Tanner, V. I. Baranov and D. R. Bandura, Reaction cells and collision cells for icp-ms: A tutorial review, *Spectrochim. Acta, Part B*, 2002, **57**(9), 1361–1452.
49 N. Yamada, J. Takahashi and K. Sakata, The effects of cell-gas impurities and kinetic energy discrimination in an octopole collision cell icp-ms under non-thermalized conditions, *J. Anal. At. Spectrom.*, 2002, **17**(10), 1213–1222.
50 J. O. Jeppsson, H. Kristensson and C. Fimiani, Carbohydrate-deficient transferrin quantified by hplc to determine heavy consumption of alcohol, *Clinical Chemistry*, 1993, **39**(10), 2115–2120.

Supplementary Material (ESI) for Metallomics


# Absolute quantification of transferrin in blood samples of harbour seals using HPLC-ICP-MS

Mechthild Grebe, Daniel Pröfrock, Antje Kakuschke, Jose A.C. Broekaert and Andreas Prange

## Sample preparation

All sample handling and manipulation was carried out under cooled conditions (4 °C) inside a class 100 clean bench, with the aim to minimise contaminations and sample degradation, respectively.

Since the absolute quantification requires a uniform iron stoichiometry of the different Tf glycoforms, an iron saturation procedure was developed based on a method described by del Castillo Busto *et al.* [27, 32]. Briefly, 100 µL serum were mixed with 5 µL of a 500 mM $Na_2CO_3$ solution as well as 5 µL of a 10 mM $FeCl_3$ solution followed by an incubation time of 30 minutes at room temperature. To improve the long term stability of the chromatographic column as well as to remove additional interfering matrix components from the serum samples, the original method was further optimised by the implementation of a lipoprotein precipitation step similar to the one described by Jeppsson *et al.* [50]. Therefore, 5 µL of the precipitation reagent (2 g $MgCl_2$ and 1 g dextransulphate in 10 mL $H_2O$) were added to the iron saturated sample. After a further incubation time of additional 30 minutes at 4 °C, the sample was centrifuged for 10 minutes with 18000 *g* at 4 °C. 100 µL of the supernatant were diluted with 400 µL of a 20 mM, pH 6.5 Bis-Tris buffer followed by an additional centrifugation step (conditions as mentioned before) for 5 minutes. The resulting supernatant was stored under cooled conditions until its further investigation. This combined sample preparation procedure was published by Grebe *et al.* [24].

**Table S-1:** Compilation of the average retention times and peak area RSDs (n=3) obtained during the repetitive, highly resolved separation of Tf glycoforms using anion exchange HPLC-ICP-MS for the certified reference serum ERM-DA470k/IFCC

| peak no. | average retention time [min] | retention time RSD [%] | peak area RSD [%] |
|---|---|---|---|
| $S_2$ | 9.36 | 0.24 | 5.59 |
| $S_3$ | 10.37 | 0.17 | 1.35 |
| $S_4$ | 11.94 | 0.24 | 1.84 |
| $S_5$ | 13.44 | 0.18 | 5.23 |
| $S_6$ | 14.93 | 0.50 | 0.48 |

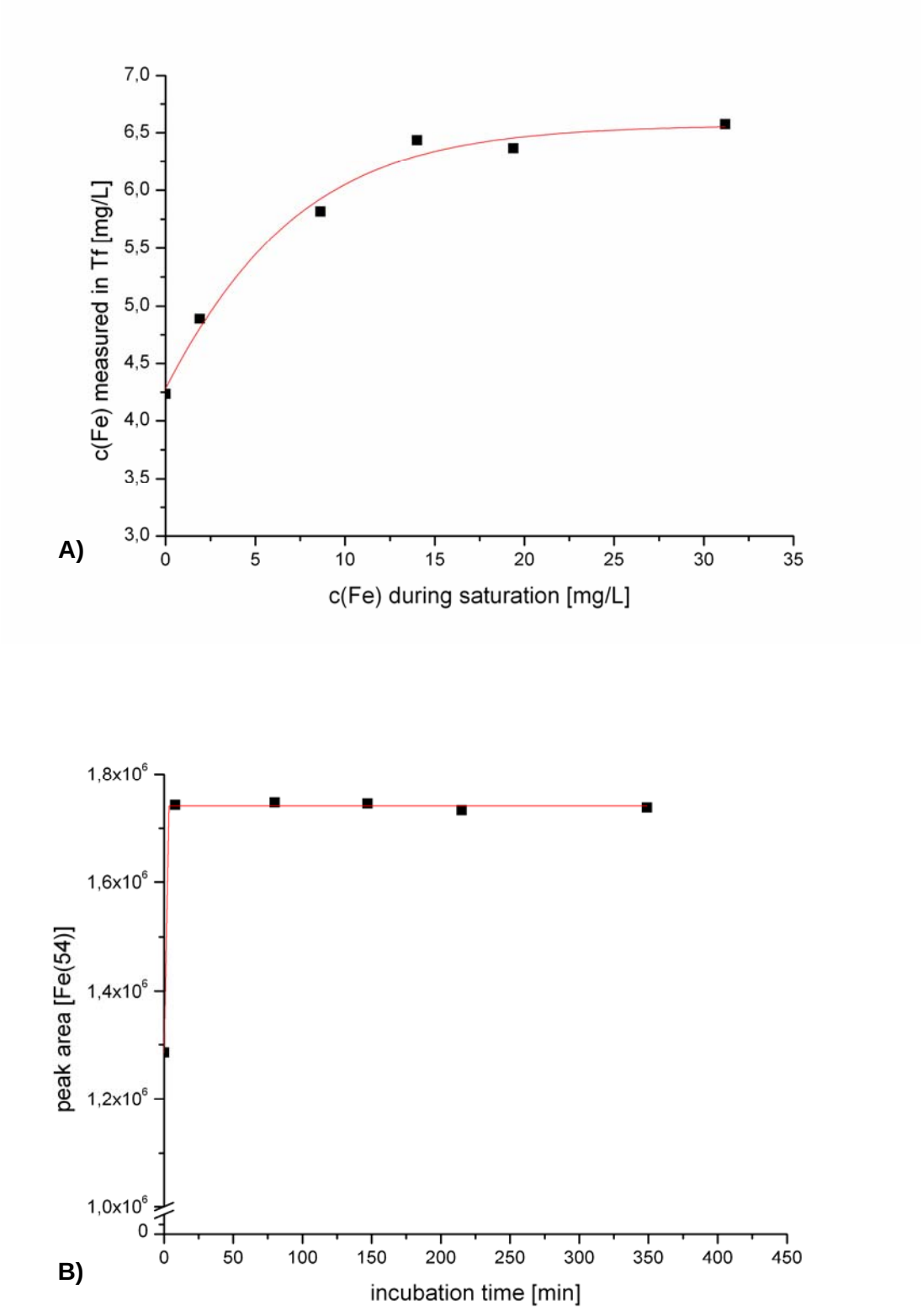


**Figure S-1**

**A)** Evaluation of the optimal iron concentration in the reaction mixture to allow an uniform iron saturation **B)** Investigation of different incubation times on the Tf iron saturation.