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PAPER

Comparison of different methods for the absolute quantification of harbour seal transferrin glycoforms using HPLC-ICP-MS†

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Marine mammals such as harbour seals (*Phoca vitulina*) represent a valuable indicator for the state of their habitat or environmental changes in particular due to their key role as top predators within the marine food web. Therefore, the characterization (qualitative and quantitative) of target proteins of such organisms can be of potential use for environmental monitoring processes. In this regard, serum transferrin (Tf) is a key protein related to iron transport and its metabolism. Thus, the present work illustrates the development and comparison of different approaches for the accurate, absolute quantification of Tf isolated from blood samples of North Sea harbour seals with the aim to use possible changes in Tf glycoform patterns as an additional parameter in extended studies, focusing on the assessment of their immune status. For this purpose, different HPLC-ICP-MS approaches have been developed, which allow the highly resolved separation and detection of up to nine different Tf glycoforms in seal blood samples in less than 30 minutes, as well as their sensitive and specific absolute quantification on the basis of their characteristic iron content. One method is based on the application of a reversed gradient sheath flow to compensate gradient related effects during the iron specific detection of Tf. Here a simple flow injection with a certified element standard was used for calibration. The second developed methodology utilizes isotope dilution analysis, which was applied for the quantification of the separated Tf glycoforms. Such accurate protein quantification methods are urgently needed in particular when aiming on comparable long term health related investigations. However, since both methods can be applied independently of the availability of specific protein standards or antibodies they can be seen as universal quantification methods for the targeted biomarker.

Introduction

Anthropogenic activities as well as the sustained direct, airborne or river based input of pollutants influence the North Sea environment, which still represents one of the most affected marine ecosystems worldwide. The quantification of pollution levels of different environmental compartments with either organic or inorganic contaminants as well as the measurement of individual body burdens of selected organisms at different levels of the food chain still represents the "standard procedure" used, to derive information on the status description of an ecosystem.[1–5] During recent years the investigation of possible biological effects related to the measured contaminant levels gained much interest, since it has been realized that the pure chemical analysis of environmental samples does not provide any effect related information. Here in particular molecular biomarkers are advantageous to those at the cellular, physiology, organism, population or ecosystem level, since their earlier response may allow fast enforcement of measures before the potentially irreversible damage of an ecosystem.[6] Top predators within the marine food web such as harbour seals (*Phoca vitulina*) can be utilized especially as indicators for medium and long term changes inside their habitat. In particular the bioaccumulation of environmental contaminants in the tissues of marine mammals is suspected to cause suppression of the immune system, which increases the vulnerability of these organisms to pathogens or infectious diseases.[7–9] Within this context different studies were already conducted, focusing on the health/immune status[10–12] and/or contaminant body burdens[13–16] as well as standard clinical chemistry parameters[17] and several acute phase proteins

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(APP)[17–19] of seals living in different regions of the Wadden Sea ecosystem. Especially APP, which are synthesised in the liver during an acute phase reaction, are interesting markers for the health status of an individual. In veterinary medicine, the analysis of APP represents a valuable diagnostic tool to address the progress of various diseases and stress at the cellular level as well as for acute pathogenic effects.[20–22]

The ongoing either methodological or instrumental developments in analytical chemistry yield a number of new approaches to further improve biomarker based environmental studies such as the utilization of proteomic/metallomic or genomic techniques.[23] The current approaches for either the qualitative or the quantitative analysis of APP in mammalian species are mainly based on assay kits or the determination of the corresponding mRNA *via* RT-PCR, however both strategies show some limitations, since different studies showed strong variabilities between the measured mRNA concentration and the corresponding real protein expression in related cell lysates.[24] In addition mRNA cannot be used to investigate post-translational modifications such as glycosylation, which represents a key event during various biological processes and which could provide additional valuable health related diagnostic information.[25] Furthermore, the applicability of immunoassay based approaches for environmental analysis focusing on marine mammals such as harbour seals is in particular limited due to the lack of suitable antibodies. The high variability of either mRNA or assay based detection and quantification approaches as well as the often poor comparability of results obtained by different labs or with different assay kits represents a further limitation in particular when focusing on long term investigations.

Therefore, new analytical approaches are urgently needed, which allow the accurate absolute quantification of important acute phase proteins as well as their post-translationally modified forms directly at the protein level. Within this context, techniques such as liquid chromatography hyphenated to inductively coupled plasma mass spectrometry (LC-ICP-MS) provide interesting possibilities for the analysis of metal containing protein biomarkers, which feature a known, stable metal stoichiometry. These properties can be easily utilized either for qualitative or for quantitative analysis. Here ICP-MS offers some unique properties, since its sensitivity depends on the mass of an analyte that enters the ion source independent of its chemical form. In consequence, simple inorganic element standards can be often used to quantify complex biomolecules such as proteins. Within the group of iron containing proteins transferrin (Tf) represents the most important diagnostic APP as well as iron-transporting metalloglycoprotein in the blood of mammals. In clinical diagnosis, altered distributions of Tf glycoforms are already used as biomarkers *e.g.* for glycosylation disorders as well as for various liver diseases.[26–28] In this context liver diseases represent one of the most frequently observed pathological disease patterns in harbour seals of the North Sea. Therefore, veterinary medicine uses Tf as a helpful diagnostic parameter in combination with other APP for assessing the progression of disease or as an indicator for the general health status of mainly domestic but also wildlife animals.[29]

In order to allow the accurate quantification of Tf glycoforms, different approaches have been developed recently based on the use of liquid chromatography for separation (HPLC) and inductively coupled plasma mass spectrometry (ICP-MS) for an iron specific detection.[30–32] In general two different quantification strategies can be applied. Since iron features different stable isotopes, isotope dilution analysis (IDA) can be applied using Fe tracers of different isotopic abundances to obtain glycoforms quantification[30] but also information about the Tf iron saturation.[33] A second straightforward way for the quantification of seal Tf glycoforms is based on a simple post-column flow injection of a certified Fe standard, while using a reversed gradient sheath flow to compensate gradient related effects on the instrumental response.[32] In the present work both analytical strategies are critically compared for the analysis of harbour seal Tf glycoforms. Overall the analysis of structural differences of selected diagnostic protein markers such as Tf as well as their absolute, glycoform specific quantification in combination with further veterinary parameters represents a new approach for the assessment of the health status of marine mammals.

Experimental section

Chemicals, standards and materials

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, Ge and Cs, as well as Na_2CO_3, $FeCl_3$ and nitric acid (65% HNO_3), were obtained from Merck (Merck Certipur/Suprapur, Darmstadt, Germany). The nitric acid was sub-boiled twice before use. $MgCl_2$, dextransulfate and Bis–Tris buffer were purchased from Fluka (Fluka, Buchs, Switzerland). A 10-fold stock solution of the Bis–Tris buffer was cleaned using a self-packed column (60 cm), which contains Chelex® 100 resin (Fluka) to reduce its trace element contamination before its final dilution to the desired concentration level. The different enriched stable iron isotopes (^{54}Fe—99.84% and ^{57}Fe—95.90%), which were used during all IDA experiments, were obtained as Fe_2O_3 from STB Isotope Germany GmbH (STB Isotope Germany GmbH, Hamburg, Germany). Argon 5.0 (99.999% purity) was used as plasma gas and Hydrogen 5.0 (99.999% purity) 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 in a clean room environment (class 1000) in a clean bench (class 100) under cold conditions (4 °C) to avoid contamination and sample degradation respectively.

Samples

The blood samples investigated have been collected during different seal catching campaigns at different sites along the North Sea coast. Physiological parameters such as size, weight, gender and age were determined during the sampling followed by blood collection from the epidural vertebral vein. During all investigations the animals were continuously under observation of two veterinarians. After finishing all medical investigations as well as the sample collection, the animals were released back into the wildlife. More detailed information related to the seal catch procedure and the biological data for the seals W 01/08 Pv–W 05/08 Pv can be found elsewhere.[17] Biological information related to the other investigated animals has been already specified in two

other papers (for seals 1–5 see Grebe *et al.*[32] and for seal A–E see Kakuschke *et al.*[34]). For validation of the different methods certified reference material of human serum (ERM®-DA470k/IFCC; European Commission, JRC, IRMM, Geel, Belgium) was used during all experiments.

Instrumentation

HPLC. An Agilent 1100 series liquid chromatography system consisting of two four channel on-line degassers, two binary LC pumps, 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 was used during all experiments.

1/16″ o.d./125 μm i.d. PEEK tubing and zero dead volume fittings (Upchurch Scientific/GAT Analysen Technik, Bremen, Germany) have been used for the connection of the different modules. All LC modules were arranged in order to achieve the lowest possible dead volume between the LC setup and the ICP-MS. A T cross has been used to allow parallel UV detection (at 280 and 460 nm respectively) as well as on-line fraction collection of the separated Tf species. Detailed chromatographic conditions as well as further instrumental settings are summarised in Table 1. The gradient conditions for the human reference serum can be found in Grebe *et al.*,[32] the second pump as well as the external six port valve were only used for the FIA based calibration approach.

ICP-MS. For sensitive element specific detection an Agilent 7500cs collision/reaction cell ICP-MS system (Agilent Technologies, Tokyo, Japan) has been used. To reduce the background, especially for ^{56}Fe, which is mainly interfered by polyatomic ions such as $^{40}Ar^{16}O^+$, H_2 with an optimum flow-rate of 5 mL min^{-1} was used as cell gas. In addition kinetic energy discrimination obtained by the settings of the octopole and quadrupole bias has been applied to further reduce the background on the main isotope of iron (^{56}Fe). A PFA 100 microconcentric nebuliser (Elemental Scientific, Omaha, Nebraska, USA) combined with a standard quartz double pass Scott spray chamber was used as the interface for the hyphenation of the HPLC system to the ICP-MS. Details of the ICP-MS settings are given in Table 2. For the characterization of the isotopically enriched spikes, possible contributions of spectral interferences of ^{54}Cr and ^{58}Ni have been corrected mathematically by measuring at *m*/*z* 53 for Cr and *m*/*z* 60 for Ni.

Table 1 Instrumental conditions for HPLC

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, 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 temperature	30 °C
DAD detection	280 nm, 460 nm

Gradient and switches of the external 6 port valve (pump 2 and valve only for FIA)

Time/min	Pump 1 [%B]	Pump 2 [%B]	Time/min	External valve
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		

Table 2 Instrumental conditions for ICP-MS

ICP-MS Agilent 7500cs	
RF power	1600 W
Carrier gas	0.91 L min^{-1}
Makeup gas	0.20 L min^{-1}
Extraction lens 1	6.5 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	^{53}Cr, ^{54}Fe, ^{56}Fe, ^{57}Fe, ^{58}Fe, ^{60}Ni, ^{72}Ge, ^{133}Cs
Dwell time	0.1 s

Instrumental setup for gradient compensation and flow injection analysis (FIA) based calibration

Details on the instrumental setup for gradient compensation with a counter current gradient sheath flow and FIA based calibration procedure as well as its application for the quantification of intact Tf glycoforms can be found in Grebe *et al.*[32]

Briefly a one point calibration *via* flow injection of a 1 mg L^{-1} iron element standard solution (as $Fe(NO_3)_3$, Merck Certipur, traceable to NIST CRM) in 2% nitric acid has been used for quantification of the different Tf glycoforms.

Instrumental setup for post-column isotope dilution analysis (IDA)

The instrumental setup for the quantification of Tf by both the post-column IDA and the "advanced" post-column IDA or the so-called double spiking IDA is shown in Fig. 1. The double spiking IDA was first developed by del Castillo Busto *et al.* for absolute Tf quantification in cerebral fluid.[31] Anion exchange chromatography hyphenated to ICP-MS was used for the separation of the Tf glycoforms as well as its element specific detection. The necessary post-column spike for the isotope dilution analysis is continuously introduced through a low volume mixing T-piece at a flow rate of 0.1 mL min^{-1} using the internal peristaltic pump of the Agilent ICP-MS system.

Preparation of the isotopically enriched spikes

The isotopically enriched iron was provided as iron-oxide powder, which has to be solubilised before its further application. Therefore, concentrated high purity sub-boiled nitric acid was added to a few milligrams of the oxide of the individual iron

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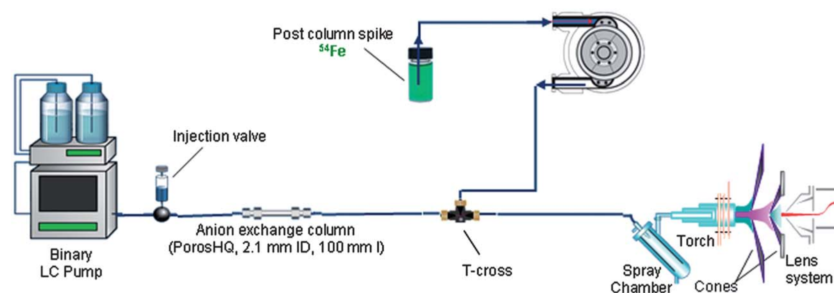

Fig. 1 The instrumental setup for the quantification of Tf for both post-column IDA and double spiking IDA.

isotopes (^{54}Fe or ^{57}Fe, respectively). The resulting solution was heated for about one hour using a temperature controlled metal free hotplate operated inside a class 100 clean bench until the total solubilisation of the metal oxide. These stock solutions were characterised by ICP-MS, measuring different mixtures of a certified standard solution of natural Fe (Merck CertiPur traceable to NIST SRM), with natural isotopic abundance of the different iron isotopes (^{nat}Fe: 5.85% ^{54}Fe, 91.75% ^{56}Fe, 2.12% ^{57}Fe and 0.28% ^{58}Fe). The following concentrations and relative isotope abundances were calculated for different enriched iron isotopes present in the two stock solutions. For the ^{57}Fe stock solution a concentration of 867.85 mg L^{-1} with isotope abundances of 0.07% ^{54}Fe, 3.75% ^{56}Fe, 95.48% ^{57}Fe, 0.71% ^{58}Fe has been measured, while the ^{54}Fe stock solution shows a concentration of 214.15 mg L^{-1} with isotope abundances of 99.12% ^{54}Fe, 0.84% ^{56}Fe, 0.02% ^{57}Fe, 0.03% ^{58}Fe. The ^{57}Fe spike (95.48% enrichment) was used for the iron saturation procedure when utilizing the double spiking IDA approach, while the ^{54}Fe spike (99.12% enrichment) diluted in 20 mM Bis–Tris buffer of pH 6.5 to a concentration of about 90 mg L^{-1} has been used for the absolute Tf quantification by both post-column IDA methods.

Sample preparation for absolute Tf quantification

Blood serum samples of seals were isolated from Serum Gel S monovettes (Sarstedt AG & Co, Nümbrecht, Germany). The sample handling and manipulation were carried out inside a class 100 clean bench under cold conditions to avoid contaminations and sample degradation if not otherwise stated. The different compared quantification methods require a uniform iron stoichiometry of the Tf molecules. Therefore, before analysis an iron saturation has been conducted as described before.[35] Briefly, 100 μL serum were mixed with 5 μL of a 500 mM Na_2CO_3 solution as well as a 25 mM $FeCl_3$ solution followed by an incubation time of 30 min at room temperature. Additionally a lipoprotein precipitation step was performed to remove additional interfering matrix components from the serum samples. After an incubation of again 30 min at 4 °C the sample was centrifuged. 100 μL of the supernatant were diluted with 400 μL of a 20 mM, pH 6.5 Bis–Tris buffer to adjust the pH, followed by an additional centrifugation step. The resulting supernatant was short-term stored under cold conditions and used for the quantification of the separated Tf glycoforms.

For the double spiking IDA the saturation process of Tf has to be adapted due to the low pH of the stock solution of the isotopically enriched ^{57}Fe spike, which contains concentrated nitric acid. Therefore, the stock solution was diluted in 20 mM Bis–Tris buffer to an iron concentration of 200 mg L^{-1}, followed by an additional dilution step using the 500 mM Na_2CO_3 solution to adjust the pH, which results in a final iron concentration of about 100 mg L^{-1}.

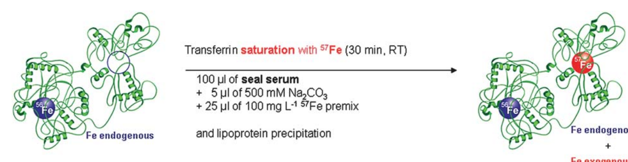

Fig. 2 Schematic illustration of the sample preparation process for the advanced IDA using the ^{57}Fe isotope.

The Tf iron saturation was performed as described above, however the 5 μl of the 25 mM $^{nat}FeCl_3$ solution was replaced by 25 μl of the 100 mg L^{-1} ^{57}Fe premix. All other steps were performed as already described for the Tf iron saturation with the $^{nat}FeCl_3$ solution. For clarifying the sample preparation process using ^{57}Fe, the procedure is schematically illustrated in Fig. 2.

Results and discussion

Quantification of seal Tf and its glycoforms with FIA

The quantification of Tf and its glycoforms with HPLC-ICP-MS using a one point calibration *via* three flow injections to calibrate each chromatogram was validated using a certified reference material of human serum (ERM®-DA470k/IFCC). With this method a Tf concentration as sum of all quantified glycoforms of 2.33 ± 0.03 g L^{-1} was calculated, which is in good agreement with the certified total Tf concentration of 2.35 ± 0.08 g L^{-1}.[32]

Fig. 3a shows, as an example, a chromatogram of a serum sample of seal D. In this case, the serum sample of seal D contained a total Tf concentration of 2.42 g L^{-1} with a standard deviation of 0.08 g L^{-1} (data presented in Table 3). The limit of detection (LOD) and the limit of quantification (LOQ) were calculated using an aliquot of serum sample of seal D, with the signal-to-noise ratio (S/N) of S/N ≥ 3 and S/N ≥ 10, respectively. The LOD for this methodology turned out to be 55.2 μg mL^{-1} Tf, while the LOQ was 184 μg mL^{-1}.

Quantification of seal Tf glycoforms using post-column IDA compared to FIA

Post-column IDA represents one of the most accurate analytical techniques for the quantitative analysis of trace metals and

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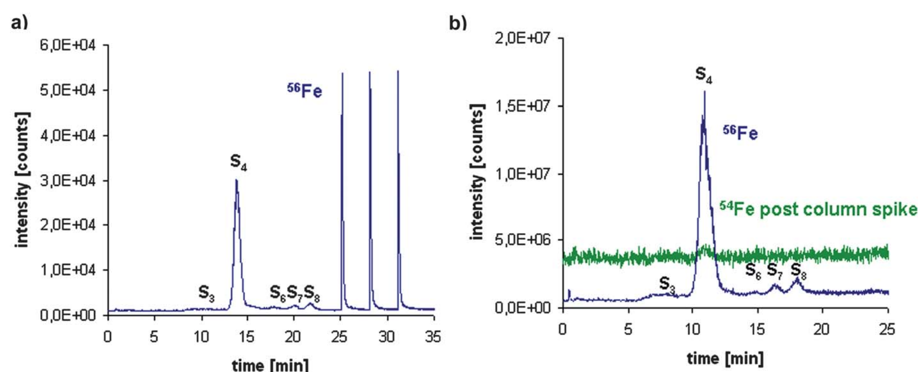

Fig. 3 Comparison of two chromatograms of a serum sample from seal D obtained by (a) FIA and (b) post-column IDA.

Table 3 Overall quantified Tf concentration with standard deviation (SD) and relative standard deviation (RSD) calculated using the FIA and post-column IDA approaches for two seal serum samples

	FIA			IDA			Difference
	Tf/ g L^{-1}	SD/ g L^{-1}	RSD [%]	Tf/ g L^{-1}	SD/ g L^{-1}	RSD [%]	Tf/ g L^{-1}
Seal C	**2.78**	0.05	1.80	**2.87**	0.05	1.84	0.09
Seal D	**2.42**	0.08	3.31	**2.50**	0.01	0.44	0.08

metalloids using ICP-MS whenever the targeted element features at least two stable isotopes. To allow the application of post-column IDA the effluent of the Poros HQ column was continuously merged with a "spike" flow, which contains the enriched ^{54}Fe post-column spike. As indicated in Fig. 1 the internal peristaltic pump of the ICP-MS has been used for the generation of a continuous spike flow, which is introduced through a mixing T, in order to allow the fast equilibration between the spike and the sample, which is essential for accurate IDA. The concentration of the ^{54}Fe isotope within the spike solution was optimised to 90 µg L^{-1}, which results in a measured ^{56}Fe/^{54}Fe isotope ratio of the baseline in the range of 0.1 to 2, which is mandatory to minimize the statistical error during IDA.[36]

A chromatogram of post-column IDA for seal Tf (seal D) is shown in Fig. 3b. Here the same sample has been measured as before with the FIA method (see Fig. 3a). The data points are smoothed using the Savitzky–Golay smoothing filter (polynomial degree of 2, 7 points left, 7 points right). The quantification of the Tf concentration with the post-column IDA is carried out by using the mass flow isotope equation (eqn (1)). The mathematical derivation of this equation is described elsewhere.[37–39] With this equation the mass flow of the sample [$MF_{sample}(t)$] can be calculated as a function of the time. This function delivers directly the mass flow of the ^{56}Fe isotope during separation, which allows the calculation of the absolute iron amount related to each separated peak, by simple integration of the peak areas. In combination with the injection volume and the dilution factor of the sample, the concentration of ^{56}Fe for each glycoform peak can be determined.

From the data of the measured chromatogram the isotope ratio as a function of the time of the natural ^{56}Fe and the post-column spike with ^{54}Fe [$R^{56/54}{}_{mixture}(t)$] can be created and used for the calculation of the mass flow as described in eqn (1).

$$\mathrm{MF}_{\mathrm{sample}}(t) = c_{\mathrm{spike}} \times \mathrm{flow}_{\mathrm{spike}} \times \frac{A_{\mathrm{r}}^{\mathrm{sample}}}{A_{\mathrm{r}}^{\mathrm{spike}}} \times \left(\frac{x_{\mathrm{spike}}^{54} \times R_{\mathrm{mixture}}^{56/54}(t) - x_{\mathrm{spike}}^{56}}{x_{\mathrm{sample}}^{56} - R_{\mathrm{mixture}}^{56/54}(t) \times x_{\mathrm{sample}}^{54}} \right) \quad (1)$$

The mass flow of the sample [MF_{sample}] equals the concentration of the ^{54}Fe post-column spike [c_{spike}] multiplied with the flow of the ^{54}Fe post-column spike [$flow_{spike}$], multiplied with the ratio of the relative atom mass [A_r] of sample to spike, multiplied with a term of the isotopic abundance [x] in sample, spike and the measured mixture.

For seal B an overall Tf concentration of 2.32 ± 0.03 g L^{-1} was calculated. This is in agreement with the measured concentration obtained with the FIA based method, while the difference of 0.10 g L^{-1} is less than the sum of the standard deviations of both methods (Table 3). The LOD was estimated to be about 101 µg mL^{-1} Tf and the LOQ of 335 µg mL^{-1}.

Measurement of the iron saturation by application of double spiking IDA

In addition to the post-column IDA, which allows absolute quantification of the different Tf glycoforms, the use of different isotopically labelled tracers permits us to obtain more information on the Tf such as the Tf saturation (percentage of Tf binding sites occupied by Fe) and that is currently measured to monitor the Fe status. For this purpose, the serum was saturated with ^{57}Fe as tracer as shown in Fig. 2 and a tracer of ^{54}Fe was used for post-column calculations similar to the work developed by del Castillo Busto *et al.*[31] for Tf analysis in human cerebral fluid.

Using a second isotopic enriched iron spike increases the complexity of the necessary mathematic calculations. Therefore, an isotope pattern deconvolution (IPD) procedure is used. The mathematic derivation of the IPD is described elsewhere.[40,41]

The iron composition of the measured sample can be described as follows in eqn (2), with A as isotopic abundance and x as molar fraction.

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$$\begin{pmatrix} A^{56}_{\text{sample}} \\ A^{57}_{\text{sample}} \\ A^{54}_{\text{sample}} \end{pmatrix} = \begin{pmatrix} A^{56}_{\text{natural}} & A^{56}_{\text{tracer}} & A^{56}_{\text{postcolumn}} \\ A^{57}_{\text{natural}} & A^{57}_{\text{tracer}} & A^{57}_{\text{postcolumn}} \\ A^{54}_{\text{natural}} & A^{54}_{\text{tracer}} & A^{54}_{\text{postcolumn}} \end{pmatrix} \times \begin{pmatrix} x_{\text{natural}} \\ x_{\text{tracer}} \\ x_{\text{postcolumn}} \end{pmatrix} \quad (2)$$

If the vector of the isotopic abundance of the sample is $\boldsymbol{y}$, the matrix of the isotopic abundance of the single components is $\boldsymbol{A}$, and the vector of the molar fraction is $\boldsymbol{x}$, then eqn (2) can be written as follows in eqn (3).

$$\boldsymbol{y} = \boldsymbol{A}\boldsymbol{x} \quad (3)$$

Since $\boldsymbol{A}$ is a regular quadratic matrix the inverse matrix $\boldsymbol{A}^{-1}$ exists and in this case eqn (4) results.

$$\boldsymbol{x} = \boldsymbol{A}^{-1}\boldsymbol{y} \quad (4)$$

Mass bias correction was carried out using the exponential function described by Rodriguez-Gonzales *et al.*[38]

For the validation of the method again the certified reference material of human serum (ERM®-DA470k/IFCC) was used. One corresponding chromatogram of the reference serum, which shows the different measured iron isotopes, is given in Fig. 4. In this case the ^{54}Fe post-column spike is used for the quantification, while the ^{57}Fe tracer was used for the saturation. The measured ^{56}Fe isotope represents the amount of iron, which is already naturally present in the different glycoforms. The quantification of the natural ^{56}Fe content allows the calculation of the natural iron saturation level of Tf.

For comparison also for this method the LOD and LOQ are determined for a serum sample of seal B. The LOD for the ^{56}Fe isotope is 88.61 mg L^{-1} seal Tf and the LOQ is 295.36 mg L^{-1} seal Tf and for the ^{57}Fe isotope they are 17.16 mg L^{-1} seal Tf and 57.21 mg L^{-1} seal Tf, respectively.

Fig. 5 shows a chromatogram from the double spiking IDA applied to a seal serum sample (seal D), which utilizes the ^{57}Fe enriched spike as tracer (red) for the Tf saturation and a ^{54}Fe enriched post-column spike (green) for absolute protein quantification. The measured serum iron concentration, the unsaturated iron binding capacity (UIBC, sites of Tf free of metal), the total iron binding capacity (TIBC, concentration of Fe present in Tf), the calculated Tf concentration and the saturation of the Tf can be obtained from this experiment. The results for 15 analysed seal serum samples are listed in Table 4.

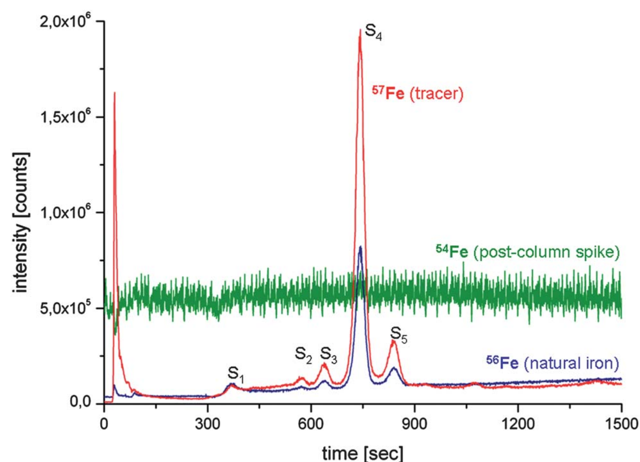

Fig. 4 Chromatogram acquired with the advanced post-column IDA method of the reference serum ERM-DA470k/IFCC® with ^{57}Fe as tracer for the Tf saturation and a ^{54}Fe post-column spike for quantification.

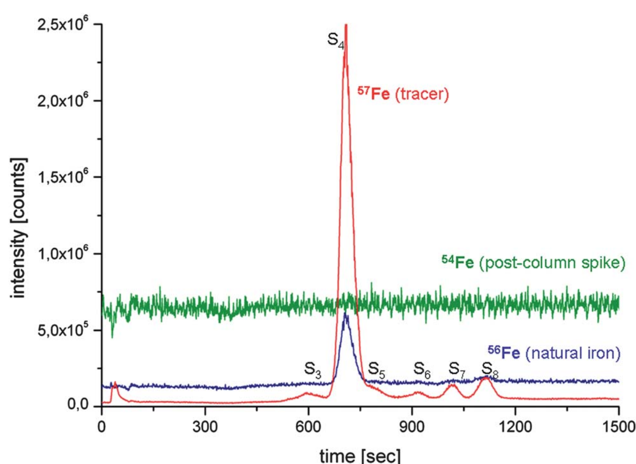

Fig. 5 Chromatogram indicating the application of the advanced post-column IDA for the analysis of a serum sample derived from seal D with ^{57}Fe as tracer for the Tf saturation and a ^{54}Fe post-column spike for quantification.

As can be observed, the total Tf levels range from 1.73 to 2.64 g L^{-1}, similar to humans, and the saturation level, although a bit more disperse (from 27 to 47%), have an average value of 37%, comparable to ~30% found in humans. On the other hand, the circulating Fe (strongly dependent on the diet) seems to be quiet homogeneous among animals with average values of about 19 µmol L^{-1} which is comparable to the values found in humans (about 17 µmol L^{-1}). Therefore, although the glycoforms distribution is completely different in seal and human serum (see Fig. 4 and 5), the Fe transporting characteristics of the protein seem to be comparable in both species.

Comparison of the double spiking IDA and FIA quantification methods for seal Tf glycoforms

The Tf concentration of 15 seals was obtained by FIA and double spiking IDA and the results were compared. Table S1 (see ESI†) shows the measured concentrations of Tf and carbohydrate-deficient transferrin (CDT represents, sum of a-, mono- and disialo-transferrin) as well as the concentration of the dominant glycoform S_4 and the relative standard deviations (SDs) obtained for both methods. To allow a better comparison the difference in the Tf, CDT and S_4 concentrations quantified with both methods has been calculated. Additionally, the correlation of the Tf, CDT and S_4 concentration results obtained with both methods is plotted in Fig. 6. Total Tf concentration results seem to be in good agreement using both strategies for 11 out of 15 and just four animals (seal 5, W02/08Pv, W04/08Pv, seal A) show important discrepancies in the Tf results. The slope of the correlation plots indicates minor differences compared to the ideal slope of the correlation line, but this is within the measuring uncertainty of both methods.

Table 4 Serum iron, unsaturated iron binding capacity (UIBC), total iron binding capacity (TIBC), Tf concentration and Tf saturation of 15 seal serum samples measured with the advanced post-column IDA approach

Sample	Serum iron ^{56}Fe (endogenous)/μmol L^{-1}	UIBC ^{57}Fe (exogenous)/μmol L^{-1}	TIBC ^{56}Fe + ^{57}Fe/μmol L^{-1}	Tf/g L^{-1}	Tf saturation [%] ^{56}Fe/TIBC × 100
Seal 1	14.57	28.62	43.19	1.75	33.74
Seal 2	11.60	31.13	42.73	1.73	27.14
Seal 3	21.08	28.50	49.57	2.01	42.52
Seal 4	26.96	38.15	65.11	2.64	41.41
Seal 5	20.12	37.84	57.96	2.35	34.71
W01/08Pv	22.51	25.41	47.92	1.94	46.97
W02/08Pv	26.78	134.28	161.06	6.53	16.63
W03/08Pv	15.74	27.25	42.99	1.74	36.58
W04/08Pv	18.88	76.19	95.07	3.86	19.92
W05/08Pv	20.90	37.57	58.46	2.37	35.74
Seal A	16.56	112.94	129.50	5.25	12.79
Seal B	18.56	40.78	59.34	2.41	31.28
Seal C	22.50	45.21	67.72	2.75	33.25
Seal D	9.30	47.87	57.17	2.32	16.28
Seal E	10.22	52.49	62.71	2.54	16.30
Average	19.05	30.95	50.00	2.03	37.73
Median	20.90	28.62	47.92	1.94	36.58
Min	11.60	25.41	42.73	1.73	27.14
Max	26.96	38.15	65.11	2.64	46.97
Number	7	7	7	7	7
5% percentile	12.49	25.96	42.80	1.74	29.12
95% percentile	25.63	37.98	63.12	2.56	45.63

Noticeably, for these four animals an elevated CDT level (FIA: 9.3%/31.1%/16.2%/15.7%) was determined (compare also with Kakuschke *et al.* Fig. 2).[17] The discrepancy is related to the differences between the determined CDT concentrations, whereas the concentration of the dominant glycoform S_4 is identical to both methods (see Fig. 6, the red squares correspond to the four animals with the elevated CDT level). For this purpose it is excluded that the CDT excess quantified with the double spiking IDA originated in the cleavage of sialic acid residues from the dominant and the higher sialinated glycoforms, since in this case the concentration of S_4 would be reduced in comparison to the S_4 concentration quantified with the FIA method.

In the serum sample of seal 2 and seal 4 also differences in the S_4 concentration, which originated in the not baseline separation of S_4 and S_5, have been observed, while the difference of the total Tf concentration from these two animals is below 0.2 g L^{-1}.

About the origin of the discrepancy of the four samples with elevated CDT levels some assumptions were made. One possibility to explain the discrepancy would be the varying affinity of the CDT glycoforms to the ^{57}Fe isotope, which was used for the Tf saturation in the double spiking IDA quantification method. Biological systems do have a minimal different affinity for the different iron isotopes,[42] but this would not explain such a strong preference of the CDT glycoforms for the ^{57}Fe compared to the ^{56}Fe isotope which has been used for the normal saturation procedure. Additionally, Hamano Nagaoka and Maitani[43] showed that the number of sialic acid residues do not influence the affinity of Tf for iron.

A comparative study of two different IDA methods for the quantification of the Tf glycoforms reflected no variation in the affinity of human Tf to the different iron isotopes.[30] But this study only compared Tf concentrations of human patients with a normal CDT level, so there are no data for the elevated CDT level. The more feasible explanation relies on the modified saturation procedure. The ^{57}Fe was dissolved in concentrated nitric acid and was diluted with the Bis–Tris buffer and Na_2CO_3 solution for pH adjustment. So there was a higher CO_3^{2-} concentration present during the saturation procedure for the double spiking IDA. For testing such possible effects during the saturation as a result of a too low concentration of the synergetic binding anion, some samples were additionally saturated with natural iron and the double concentrated Na_2CO_3 solution. The Tf and CDT concentration of these samples, which have been prepared with a two times higher CO_3^{2-} concentration and which have been quantified using the FIA based method, showed identical results compared to the normal saturated samples. One additional possibility to check if the saturation is completed will be to add directly bicarbonate to the saturation process instead of carbonate, similar to del Castillo Busto and co-workers, who used bicarbonate during the saturation step.[44] A third possible explanation for the overestimated CDT level by the double spiking IDA could be that there are other ^{57}Fe species which co-elute from the column through the different pH conditions with the CDT glycoforms. For the ^{57}Fe there is also a nonretarded peak after the injection (see Fig. 4) whereas serum samples saturated with natural iron as necessary for the FIA approach do not show an iron peak at this time. However, if there are other ^{57}Fe species, they also would elute at the retention time of the CDT glycoforms in samples with no CDT. In consequence, also for these samples there would be a false positive elevated CDT level detected. The quantification of the eleven samples with the normal CDT level delivers identical Tf and CDT concentrations for both methods, so this is also not a suitable explanation. A fourth possibility could be the co-elution of Cd species together with the separated CDT glycoforms. Cd^{++} ions (^{114}Cd 28.73%) would interfere with the detection of the mass 57. For excluding this possible effect the ^{57}Fe spike was checked for Cd contaminations by ICP-MS, but no Cd was present within the spike solution. All made assumptions for the differences in the

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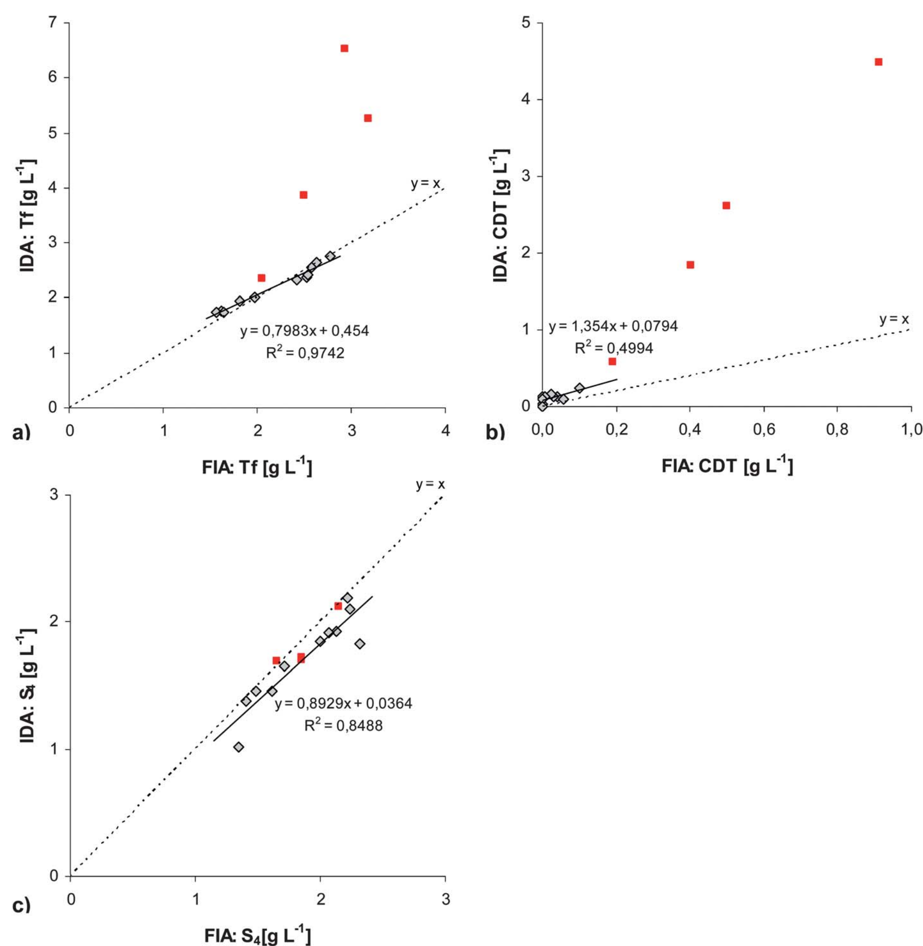

Fig. 6 Correlation plots of (a) Tf concentration, (b) CDT concentration and (c) the concentration of the dominant glycoform S_4 obtained by FIA and double spiking IDA. The red squares are the data of the four animals with the elevated CDT level.

concentrations of the CDT glycoforms quantified by FIA and double spiking IDA were disproved experimentally. The most valuable explanation is that the concentration of bicarbonate is the limiting factor. Unfortunately, the overestimated CDT level for some samples obtained by the double spiking IDA could currently not be completely explained.

Conclusion

New clinical as well as environmental related investigations focusing on the diagnostic utilization of selected protein biomarkers such as transferrin urgently rely on the future availability of fast and accurate absolute quantification methods, which provide traceable and therefore long time comparable results. Here the application of HPLC-ICP-MS represents a valuable quantification approach, which in general 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.

All compared methods were suited in order to provide accurate glycoform specific, quantitative results. The simplicity of the FIA based method made it in particular interesting for the routine analysis of Tf glycoforms *e.g.* as conducted in clinical routine labs if two pumping systems are available. On the other hand, the IDA based approaches are more laborious due to the necessary additional measurements and calculations. This includes in particular mass bias correction, isotope spike characterization, spike flow characterization as well as the final complex calculations in order to transfer the measured isotope ratios into mass flow chromatograms and quantitative protein amounts respectively, especially when using the double spiking IDA approach. However, the approach with the three isotopes provides much more clinically relevant information on the samples beside the different glycoform concentrations. Among others, Fe homeostasis related parameters of interest such as serum iron, Tf saturation and TIBC can be obtained in a single chromatographic run.

In comparison to enzymatic or immunoassay based approaches for the quantification of Tf the application of HPLC-ICP-MS is in general advantageous since it can be utilized 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 mostly cannot be obtained with such methods.

Future work will focus on the utilization of these quantitative, glycoform specific information to allow the accurate investigation of differences in the individual Tf glycoform patterns, when

comparing a larger set of samples derived from different animal groups (male *vs.* female, different age, origin). Within this background also the investigation of micro-heterogeneities in the glycostructure of selected Tf glycoforms observed in abnormal glycoform patterns, which may provide additional information within the context of other parameters regarding the immune status of the investigated marine mammals, will be conducted. In addition downscaling of the used chromatographic setup in order to reduce the necessary flow rates and the solvent amount that is introduced into the plasma is anticipated, in order to improve the transport efficiency of the nebuliser and in consequence to further improve the sensitivity of the setup.

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