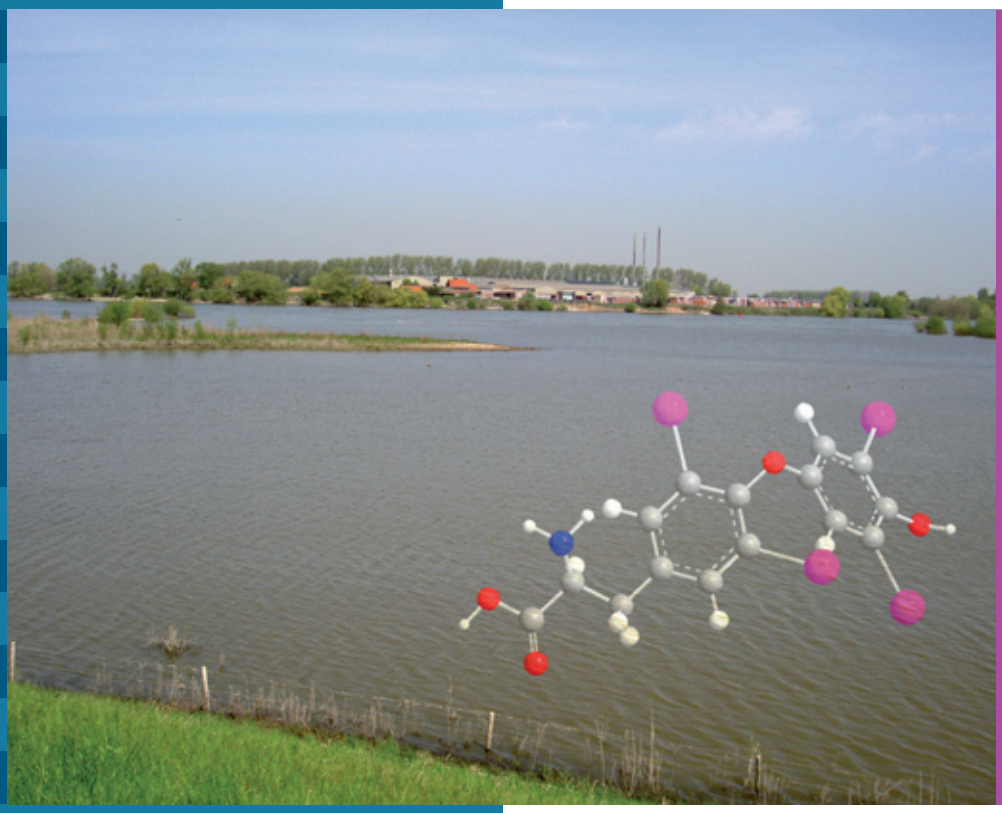
Thyroid Hormone-like Activity

Biosensor screening of surface water

RIWA
Rhine Water Works
The Netherlands



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Association of River Waterworks – RIWA

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1

Introduction and background

Introduction and background

In the scope of water quality monitoring in the Netherlands much research is put into de development of new advanced methods. In recent years, attention was focussed at pollutants showing endocrine disrupting activity. Such activities may involve several hormonal systems in various lower and higher organisms. Worldwide most of the investigations were engaged in estrogenic activities and assays were developed and used for the assessment of such endocrine activity and the effects on organisms. However, there is more than one hormonal system in humans that affects the whole organism and comprises delicate equilibriums in endogenous hormone levels. The main hormonal systems in humans are: a) those of estrogens/androgens; b) the thyroids; and c) the corticosteroids. In order to be able to assess a hormonal activity of any sample, a relevant bioassay has to be available. Estrogenic hormone disrupting (EDC) compounds have been the subject of numerous studies and today several commercial assays are available. However, studies into substances exhibiting thyroidal activity are much more limited and commercial bioassays are not yet available. In clinical chemistry the measurement of thyroid hormones and corresponding binding proteins and receptors is used for the diagnosis of thyroid-related diseases and disorders. Such assays are based on either antibodies or radiolabeled tracers. In analogy, radioligand binding assays have been developed and used for the assessment of a putative thyroidal activity of a series of environmental pollutants, such as PCBs, dioxins, etc. [1-3]. In view of the overall effect of disturbance of the thyroidal system, such as metabolism and well-being, there is a need for non-radioactive bioassays for the assessment of thyroidal activity of contaminants, in particular in water environments.

For drinking water companies using surface water as a source it is of paramount importance to know the quality developments of the source water. Such knowledge cannot be solely based on classical chemical analyses (such as heavy metals and organic micropollutants) and microbiological contamination. Rather, the multitude of potentially occurring contaminants asks for effect-oriented measurements as well.

Therefore, the Association of Rhine Water works in the Netherlands (RIWA-Rhine), included in its source water monitoring program a series of measurements for thyroidal activity in addition to existing bioassays for estrogenic activity.

As is known from the field of clinical chemistry, the main thyroidal hormones are 3,3',5-triiodothyronine (T_3) and 3,3',5,5'-tetraiodo-L-thyronine (L-thyroxine (T_4). These are synthesized in the thyroid gland as well as, to a lesser extent, peripherally and are transported in blood to their target organs bound to three proteins: thyroxine binding globuline (TBG), transthyretin (TTR) and albumin. The thyroidal status of a person is determined by the equilibrium between protein-bound and free T_3/T_4 in blood. Any disturbance may affect that status and lead to diseases and disorders. Based on the concept that various pollutants may mimic endogenous thyroid hormones, a biosensor assay was developed using TBG or TTR as biospecific binding molecules and T_4 as a ligand. Following validation of this biosensor assay, several surface water samples were processed and analysed. The results of this investigation are described in this report.

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2 Materials and methods

Biosensor assay

The development and validation of the biosensor assay for thyroidal activity has been published elsewhere [4]. This article describes the development of T4-derivatives, the design of the biosensor assay on the Biacore®, optimisation of the assay conditions using T3 and T4 as ligands, and the performance of several relevant testcompounds. The optimised biosensor assay was used in this study. In short, L-thyroxine (T4) derivative was covalently linked to the sensor chip surface without changing its affinity properties for binding thyroid binding globulin (TBG) or recombinant transthyretin (rTTR). This allowed the measurement of the binding between TBG and rTTR to the immobilized T4 by monitoring the change in mass on the chip's surface. The assays in this competitive inhibition format were performed as follows: the corresponding binding protein (18.2nM) was automatically mixed with the analyte under evaluation and injected for 120 s over the T4-derivatized sensor chip surface. Given an interaction between the analyte and the binding protein, the signal obtained inversely correlated with increasing analyte concentrations. The binding was determined in response units (RU). Initially, a series of PCBs, PHAHs was analysed to assess the applicability of the bioassay. Subsequently, several other compounds were tested, including industrial substances and pharmaceuticals and a library was set up.

Samples

Surface water was sampled at several sites, including the point-of-entry of the Rhine river into the Netherlands (Lobith), and two intake sites for drinking water production (Nieuwegein and Andijk, respectively). Three series of samples were supplied: a) from January - April, 2005, from April - July, 2005, and from August - December, 2005. Much attention was paid to the blanc sample (Evian water) with regard to reproducibility and sensitivity. The samples were extensively processed as described below and were sent to the Rikilt (Wageningen) in the form of concentrated extracts where they were measured.

Sample pretreatment

Labware was extensively cleaned before use. Samples (1 liter) were first filtrated through one or more 0.45 µm filters (Sartorius Nitrate Filters, # 13906-50-ACN) in vacuo. The filtrated samples were then extracted in a separating funnel (2 L) using 200 ml, 50 ml, and 50 ml ethylacetate, respectively. The extract was collected in a 500 ml Erlenmeyer with glass NS stop, anhydrous ethylacetate was added and vigorously shaken and remaining water was removed. Blanc sample, Evian water, was processed similarly. The extract was transferred into a Kuderna Danish assembly and evaporated to a volume of 5 tot 10 ml. In the case of larger volumes of extract, the extract was distilled. Then the extract was evaporated at 56°C under a flow of pure nitrogen to 0.5 ml. The residue was transferred into vials (Brown Autosamples Vial 1.0 ml, size 12x32 mm, conical; Omnilabo # 268462, Breda) provided with a polypropylene screw cap and PTFE/butylrubber septum containing 0.5 ml purified ethylacetate. Subsequently, the content of the vial was evaporated under nitrogen at 56°C to 2 - 3 µl and then to dryness spontaneously. The vial was sealed and DMSO was added (50 µl per 1 liter original volume).

3 Results

Standard curve

T_4 in serial dilutions was used to generate standard curves with both TTR and TBG. An example of both standard curves is given in Figure 1.a and 1.b, respectively.

Figure 3.1a: Standard curve of T4 in rTTR assay

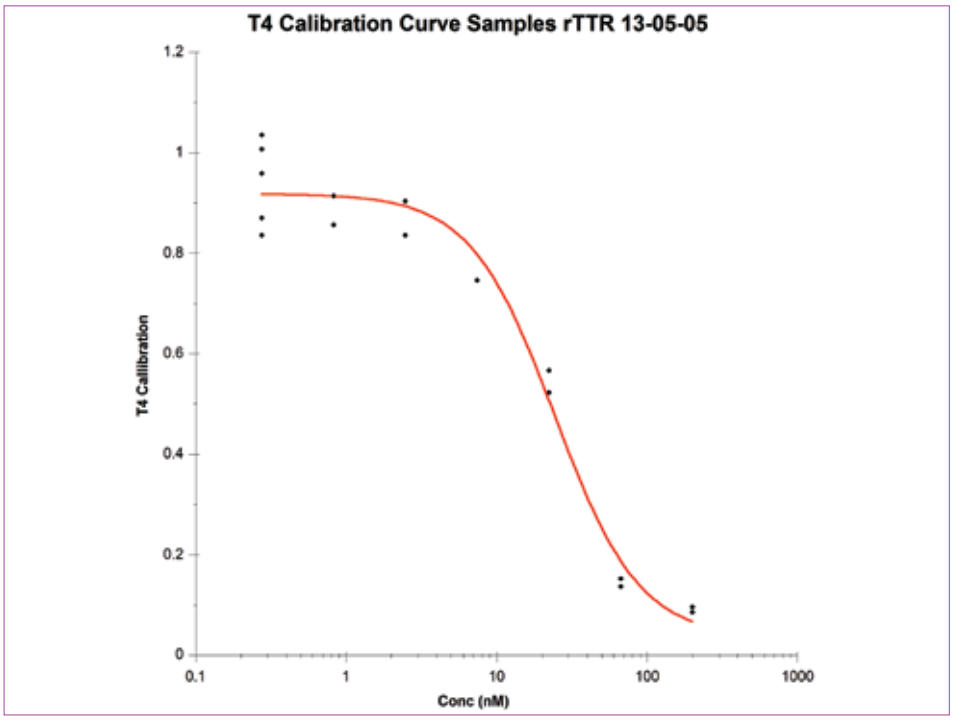
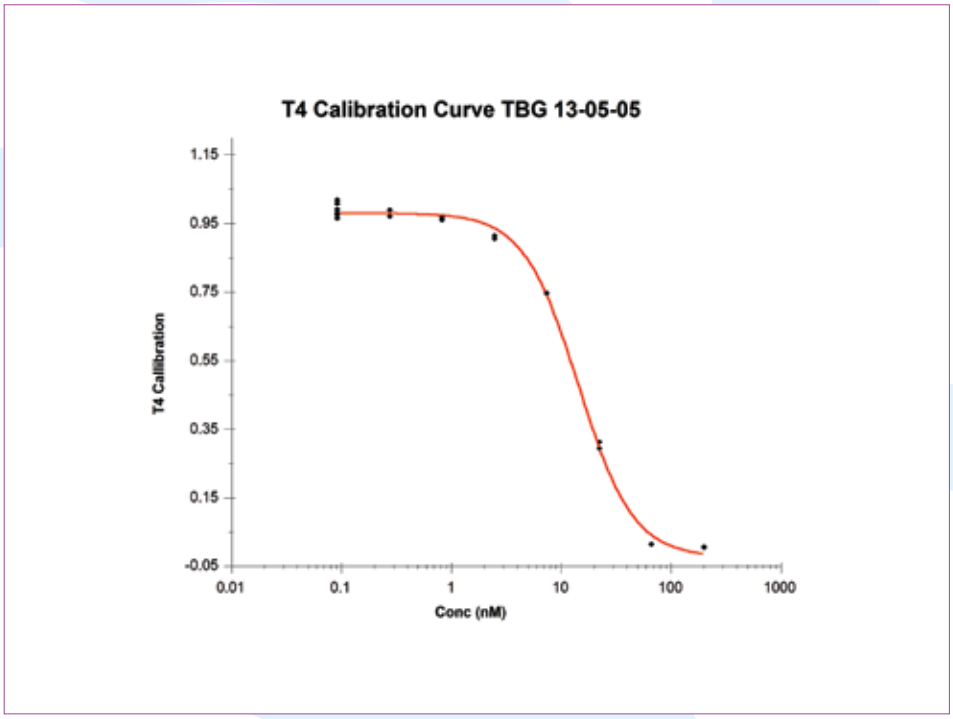


Figure 1.b: Standard curve of T4 in TBG assay.



From the standard curves the detection limit (LOD; calculated at 3 SD from standard zero) and the IC_{50} were determined. These were 5.79 nM and 22.4 nM, respectively, in the TTR assay, and 4.60 nM and 13.7 nM, respectively, in the TBG assay.

After receiving the samples in three series, these were measured in the sensor assay in small series together with standards and blancs. Each series of samples was preceded and followed by a standard curve and Evian water was included several times for control and background assessment. The results, expressed as RU, were calculated using Bioevaluation Software (Biacore™) and expressed in B/Bo as well as T4 equivalents as shown in Appendices 2 and 3 and depicted in Figure a – d.

Figure a and b present the B/Bo zero values for all the samples. In order to assess a thyroid disrupting activity above background, only those samples giving a binding value of more than 3 x SD of Evian water were considered as positive. These samples are shown in Figure c and d, expressed as T4 equivalents.

4 Discussion

Discussion

The report describes for the first time the simultaneous measurement of real surface water samples for thyroidal activity using both rTTR and TBG as binding proteins in a competitive assay on a Biacore®, biosensor device. The assay was designed by coating a T_4-derivative onto a CM5 sensor chip. Several derivatives were synthesized and tested as published.

From Figure c and d it is obvious that several samples show potential thyroidal activity because they exhibit an affinity for TTR and/or TBG. That means that they are able to displace T_3 and/or T_4 from their transport proteins in blood, leading to a transient increase in the free concentration of these thyroid hormones and consequently an increased biological activity or higher clearance. The results of the measurements are expressed as T_4-equivalents, a sum parameter. As such the surface water samples in the TTR-assay showed values between zero/Evian-water (low) and 364 nM of T_4-eq., in the TBG-assay between zero/Evian-water (low) and 183 nM of T_4-eq. In healthy persons the concentrations of total T_4 range from 5 – 12 µg/dL (around 113.3 nM) and total T_3 from 70 – 200 ng/dL (around 2.0 nM); concentrations of free T_4 range from 150 – 360 ng/dL (around 14.4 pM) and of free T_3 from 2.1 – 6 ng/dL (around 5.7 pM) [5,6]. It will be obvious that the total load of substances with thyroid-like activity in the surface water samples is higher than the free as well as the total endogenous levels of T_4 and T_3. Surprisingly, a relatively great number of samples showed affinity for TBG in the biosensor assay, while in the preliminary investigation TBG appeared predominantly specific for T_3 and T_4. From the above results no unambiguous conclusions can be drawn regarding the relation between response in the biosensor assays and the causative substances. Similarly, no relation can be found between sampling site or sampling date. The constituent compounds and their identity have to be determined by other methods, such as HPLC or GC and MS, followed by re-assay of the individual compounds or fractions for thyroidal activity. In the initial study (4) some polyhalogenated phenols, bisphenol A, polyhalogenated bisphenol A and one hydroxylated PCB (4-OH-PCB 14) showed relatively high affinities for TTR (more than 50 % with respect to T_4), of which those for tetrabromoBPA and 4-OH-PCB 14 being even much higher. Comparable results were found for several alkylphenols and BPAs in our preliminary study to transfer the biosensor assay onto microtiter plates with fluorescent detection. With regard to such phenols it has been reported that they have been detected in drinking water due to diffusion from plastic tubings. Further, it has been demonstrated that various hydroxylated metabolites generally exhibit higher thyroidal activity than their parent compounds (to be published). In the living body, ingested pollutants are partly degraded by hydroxylation, which means that the effect on thyroid status may even be much higher than calculated from the assay results. To come to a more realistic view of thyroid disrupting activity of water samples, it is suggested to submit them to an in vitro hydroxylation system (e.g. microsomal enzymes) before analysis in the biosensor assay.

The regular source water monitoring program as operated by RIWA-Rhine includes both an effect-oriented measurement of endocrine activity (using the Calux test) and, notably for the year 2005, an extensive GCMS screening at the same sampling sites as were used for the thyroid assay. Those results will be published elsewhere, and may provide a unique opportunity for comparisons between individual organic pollutants and two different sets of endocrine activity test results in the same samples.

5 Recommendations

Recommendations

Based on the results of this study it is recommended to:

- extend the measurement of surface water for thyroid disrupting activity at sewage treatment effluent sites in order to find potential sources of input into surface water;
- include a LC-MS step for those samples that score positive in the TTR-based and/or TBG-based biosensor assay to assess the identity of the constituent compounds;
- include a hydroxylation step in order to obtain a more biological relevant value for thyroidal activity;
- investigate the degree of purification of the identified components in the conventional purification process for the production of drinking water;
- investigate the source of industrial compounds found in the fractions showing thyroid disrupting activity in order to be able to take measures for reducing this activity in surface water.

6 References

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APPENDIX 1

Overview of sample sites and dates, in % T_4 inhibition (B/Bo) and STD in the TTR and the TBG sensor assays

rTTR-Based Assay Location	% T_4 Inhibition (B/Bo)	St dev	Date
Andijk 17-01-2005	0,959787	0,012012	17-01-2005
Andijk 16-02-2005	0,949965	0,014594	16-02-2005
Andijk 14-03-2005	0,716235	0,006086	14-03-2005
Andijk 13-04-2005	0,723106	0,00273	13-04-2005
Andijk 09-05-2005	0,792177	0,098678	09-05-2005
Andijk 08-06-2005	1,035143	0,125222	08-06-2005
Andijk 04-07-2005	0,950802	0,051599	04-07-2005
Andijk 01-08-2005	0,819576	0,090259	01-08-2005
Andijk 31-08-2005	**0,380274**	**0,016585**	**31-08-2005**
Andijk 26-09-2005	0,657503	0,05971	26-09-2005
Andijk 24-10-2005	0,727926	0,053524	24-10-2005
Andijk 21-11-2005	**0,40066**	**0,059176**	**21-11-2005**
Andijk 21-12-2005	0,624156	0,0205	21-12-2005
Lobith 19-01-2005	0,915278	0,000694	19-01-2005
Lobith 16-02-2005	0,611926	0,017834	16-02-2005
Lobith 20-04-2005	0,631716	0,013229	20-04-2005
Lobith 11-05-2005	**0,585949**	**0,038082**	**11-05-2005**
Lobith 08-06-2005	1,130468	0,119355	08-06-2005
Lobith 06-07-2005	1,058115	0,077135	06-07-2005
Lobith 03-08-2005	0,835538	0,131778	03-08-2005
Lobith 31-08-2005	**0,53478**	**0,017146**	**31-08-2005**
Lobith 28-09-2005	1,038335	0,084122	28-09-2005
Lobith 26-10-2005	1,152645	0,109875	26-10-2005
Lobith 23-11-2005	0,895606	0,011771	23-11-2005
Lobith 21-12-2005	0,864826	0,052806	21-12-2005
Nieuwegein (Ruw) 19-01-2005	**0,542663**	**0,017812**	**19-01-2005**
Nieuwegein (Ruw) 16-02-2005	0,740594	0,01429	16-02-2005
Nieuwegein (Ruw PNGOW-02) 16-03-2005	0,814475	0,015439	16-03-2005
Nieuwegein 13-04-2005	0,939494	0,017486	13-04-2005
Nieuwegein 11-05-2005	**0,561855**	**0,079245**	**11-05-2005**
Nieuwegein 08-06-2005	0,920638	0,104188	08-06-2005
Nieuwegein 06-07-2005	1,210139	0,095643	06-07-2005
Nieuwegein 03-08-2005	**0,397682**	**0,018666**	**03-08-2005**
Nieuwegein 31-08-2005	0,909795	0,078426	31-08-2005
Nieuwegein 28-09-2005	0,9822	0,035367	28-09-2005
Nieuwegein 26-10-2005	0,943402	0,031222	26-10-2005

Bold: values exceeding 3 x SD of the blank as described in text.

rTTR-Based Assay Location	% T_4 Inhibition (B/Bo)	St dev	Date
Nieuwegein 23-11-2005	0,665149	0,018014	23-11-2005
Nieuwegein 19-12-2005	**0,592272**	**0,004148**	**19-12-2005**
Evian 20-01-2005	1,122291	0,010616	20-01-2005
Evian 21-02-2005	1,018646	0,000395	21-02-2005
Evian 03-2005	0,848222	0,003653	??-3-2005
Evian 11-04-2005	1,010842	0,008926	04-11-2005
Evian 23-04-2005	0,581645		23-04-2005
Evian 14-06-2005	1	0,061003	14-06-2005
Evian 05-07-2005	1,044105	0,071575	07-05-2005
Evian 12-08-2005	0,955895	0,03569	08-12-2005
Evian 05-09-2005	1	0,084557	09-05-2005
Evian 06-10-2005	1,027213	0,079911	10-06-2005
Evian 03-11-2005	0,972787	0,006241	11-03-2005
Evian 22-12-2005	1	0,030501	22-12-2005
TBG-Based Assay **Location**	**% T_4 Inhibtion (B/Bo)**	**St dev**	**Date**
Andijk 17-01-2005	0,749716	0,006106	17-01-2005
Andijk 16-02-2005	0,817067	0,010177	16-02-2005
Andijk 14-03-2005	0,742506	0,00826	14-03-2005
Andijk 13-04-2005	0,779221	0,02069	13-04-2005
Andijk 09-05-2005	0,838401	0,008023	09-05-2005
Andijk 08-06-2005	0,852772	0,03313	08-06-2005
Andijk 04-07-2005	0,851351	0,025624	04-07-2005
Andijk 01-08-2005	0,794024	0,005408	01-08-2005
Andijk 31-08-2005	0,641998	0,034643	31-08-2005
Andijk 26-09-2005	0,635603	0,068703	26-09-2005
Andijk 24-10-2005	1,20319	0,055662	24-10-2005
Andijk 21-11-2005	**0,44431**	**0,008468**	**21-11-2005**
Andijk 21-12-2005	**0,395344**	**0,001352**	**21-12-2005**
Lobith 19-01-2005	0,738689	0,017308	19-01-2005
Lobith 16-02-2005	0,754865	0,016532	16-02-2005
Lobith 20-04-2005	0,646702	0,016809	20-04-2005
Lobith 11-05-2005	**0,586686**	**0,020069**	**11-05-2005**
Lobith 08-06-2005	1,129951	0,00258	08-06-2005
Lobith 06-07-2005	0,877839	0,004774	06-07-2005
Lobith 03-08-2005	0,728076		03-08-2005
Lobith 31-08-2005	0,947044	0,071322	31-08-2005
Lobith 28-09-2005	0,957942	0,057074	28-09-2005
Lobith 26-10-2005	0,749148		26-10-2005
Lobith 23-11-2005	0,727242	0,009094	23-11-2005
Lobith 21-12-2005	**0,47549**	**0,002587**	**21-12-2005**
Nieuwegein (Ruw) 19-01-2005	0,678417	0,017373	19-01-2005
Nieuwegein (Ruw) 16-02-2005	0,767129	0,008428	16-02-2005

Bold: values exceeding 3 x SD of the blank as described in text.

TBG-Based Assay Location	% T_4 Inhibtion (B/Bo)	St dev	Date
Nieuwegein (Ruw PNGOW-02) 16-03-2005	0,768056	0,021666	16-03-2005
Nieuwegein 13-04-2005	0,737826	0,019161	13-04-2005
Nieuwegein 11-05-2005	0,67443		11-05-2005
Nieuwegein 08-06-2005	0,773385	0,002612	08-06-2005
Nieuwegein 06-07-2005	0,961189	0,037147	06-07-2005
Nieuwegein 03-08-2005	**0,395427**	**0,006615**	**03-08-2005**
Nieuwegein 31-08-2005	0,848742	0,059939	31-08-2005
Nieuwegein 28-09-2005	1,119164	0,092569	28-09-2005
Nieuwegein 26-10-2005	0,987249		26-10-2005
Nieuwegein 23-11-2005	0,762196	0,001069	23-11-2005
Nieuwegein 19-12-2005	0,629439	0,01921	19-12-2005
Evian 20-01-2005	1,011633	0,047831	20-01-2005
Evian 21-02-2005	1,052291	0,016352	21-02-2005
Evian 03-2005	0,997182	0,013404	??-3-2005
Evian 11-04-2005	0,938893	0,0143	04-11-2005
Evian 23-04-2005	0,925458	0,018815	23-04-2005
Evian 14-06-2005	1,074542	0,00825	14-06-2005
Evian 05-07-2005	1,0446	0,003468	07-05-2005
Evian 12-08-2005	0,9554	0,0354	08-12-2005
Evian 05-09-2005	1	0,026457	09-05-2005
Evian 06-10-2005	0,972921	0,096018	10-06-2005
Evian 03-11-2005	1,027079	0,060476	11-03-2005
Evian 22-12-2005	1	0,001228	22-12-2005

Bold: values exceeding 3 x SD of the blank as described in text.

APPENDIX 2

Overview of sampling sites and dates, expressed in concentration of T4-equivalents (nM) in the TTR and TBG biosensor assay

TTR-based assay Location	Thyroxine Equivalents (nM T4)	STD	Date
Andijk 17-01-2005	LOW		17-01-2005
Andijk 16-02-2005	LOW		16-02-2005
Andijk 14-03-2005	111,0	2,80	14-03-2005
Andijk 13-04-2005	107,8	1,25	13-04-2005
Andijk 09-05-2005	68,9	34,67	09-05-2005
Andijk 08-06-2005	18,9		08-06-2005
Andijk 04-07-2005	17,2	15,97	04-07-2005
Andijk 01-08-2005	59,1	30,14	01-08-2005
Andijk 31-08-2005	364,5	20,02	31-08-2005
Andijk 26-09-2005	68,5	10,59	26-09-2005
Andijk 24-10-2005	LOW		24-10-2005
Andijk 21-11-2005	187,2	36,64	21-11-2005
Andijk 21-12-2005	90,4	6,16	21-12-2005
Lobith 19-01-2005	5,7	0,98	19-01-2005
Lobith 16-02-2005	161,8	9,31	16-02-2005
Lobith 20-04-2005	151,6	6,69	20-04-2005
Lobith 11-05-2005	161,8	23,37	11-05-2005
Lobith 08-06-2005	LOW		08-06-2005
Lobith 06-07-2005	LOW		06-07-2005
Lobith 03-08-2005	54,9	43,37	03-08-2005
Lobith 31-08-2005	232,5	10,63	31-08-2005
Lobith 28-09-2005	18,7	12,01	28-09-2005
Lobith 26-10-2005	51,7		26-10-2005
Lobith 23-11-2005	25,5	2,44	23-11-2005
Lobith 21-12-2005	32,0	11,05	21-12-2005
Nieuwegein (Ruw) 19-01-2005	200,4	10,68	19-01-2005
Nieuwegein (Ruw) 16-02-2005	99,9	6,50	16-02-2005
Nieuwegein (Ruw PNGOW-02) 16-03-2005	66,1	7,22	16-03-2005
Nieuwegein 13-04-2005	LOW		13-04-2005
Nieuwegein 11-05-2005	180,6	53,82	11-05-2005
Nieuwegein 08-06-2005	26,2	32,39	08-06-2005
Nieuwegein 06-07-2005	LOW		06-07-2005
Nieuwegein 03-08-2005	342,8	28,86	03-08-2005
Nieuwegein 31-08-2005	71,9	27,90	31-08-2005
Nieuwegein 28-09-2005	LOW		28-09-2005

LOW: too low to be quantified

TTR-based assay Location	Thyroxine Equivalents	STD	Date
Nieuwegein 26-10-2005	13,4		26-10-2005
Nieuwegein 23-11-2005	78,7	4,94	23-11-2005
Nieuwegein 19-12-2005	100,3	1,35	19-12-2005
TBG-Based Assay **Location**	**Thyroxine Equivalents** **(nM T4)**	**STD**	**Date**
Andijk 17-01-2005	72,0	0,67	17-01-2005
Andijk 16-02-2005	56,3	2,40	16-02-2005
Andijk 14-03-2005	73,6	1,92	14-03-2005
Andijk 13-04-2005	65,1	4,79	13-04-2005
Andijk 09-05-2005	39,1	1,15	09-05-2005
Andijk 08-06-2005	37,0	4,83	08-06-2005
Andijk 04-07-2005	37,3	3,72	04-07-2005
Andijk 01-08-2005	45,4	0,76	01-08-2005
Andijk 31-08-2005	67,4	5,29	31-08-2005
Andijk 26-09-2005	68,5	10,58	26-09-2005
Andijk 24-10-2005	LOW		24-10-2005
Andijk 21-11-2005	170,0	2,15	21-11-2005
Andijk 21-12-2005	183,1	0,39	21-12-2005
Lobith 19-01-2005	74,5	4,03	19-01-2005
Lobith 16-02-2005	70,8	3,83	16-02-2005
Lobith 20-04-2005	96,5	4,17	20-04-2005
Lobith 11-05-2005	76,1	3,29	11-05-2005
Lobith 08-06-2005	LOW		08-06-2005
Lobith 06-07-2005	33,4	0,71	06-07-2005
Lobith 03-08-2005	54,7		03-08-2005
Lobith 31-08-2005	20,5	14,19	31-08-2005
Lobith 28-09-2005	18,7	12,01	28-09-2005
Lobith 26-10-2005	51,8		26-10-2005
Lobith 23-11-2005	108,8	1,87	23-11-2005
Lobith 21-12-2005	162,3	0,62	21-12-2005
Nieuwegein (Ruw) 19-01-2005	88,8	4,19	19-01-2005
Nieuwegein (Ruw) 16-02-2005	67,9	1,95	16-02-2005
Nieuwegein (Ruw PNGOW-02) 16-03-2005	67,7	5,02	16-03-2005
Nieuwegein 13-04-2005	74,7	4,45	13-04-2005
Nieuwegein 11-05-2005	62,5		11-05-2005
Nieuwegein 08-06-2005	48,3	0,37	08-06-2005
Nieuwegein 06-07-2005	18,8	7,69	06-07-2005
Nieuwegein 03-08-2005	114,3	1,66	03-08-2005
Nieuwegein 31-08-2005	37,5	8,73	31-08-2005
Nieuwegein 28-09-2005			28-09-2005
Nieuwegein 26-10-2005	13,4		26-10-2005
Nieuwegein 23-11-2005	101,6	0,23	23-11-2005
Nieuwegein 19-12-2005	128,7	3,93	19-12-2005

LOW: too low to be quantified

APPENDIX 3

Overview of the results of the rTTR (Figure a) and TBG (Figure b) sensor assays, respectively, for all the samples. Data are expressed as B/Bo value.

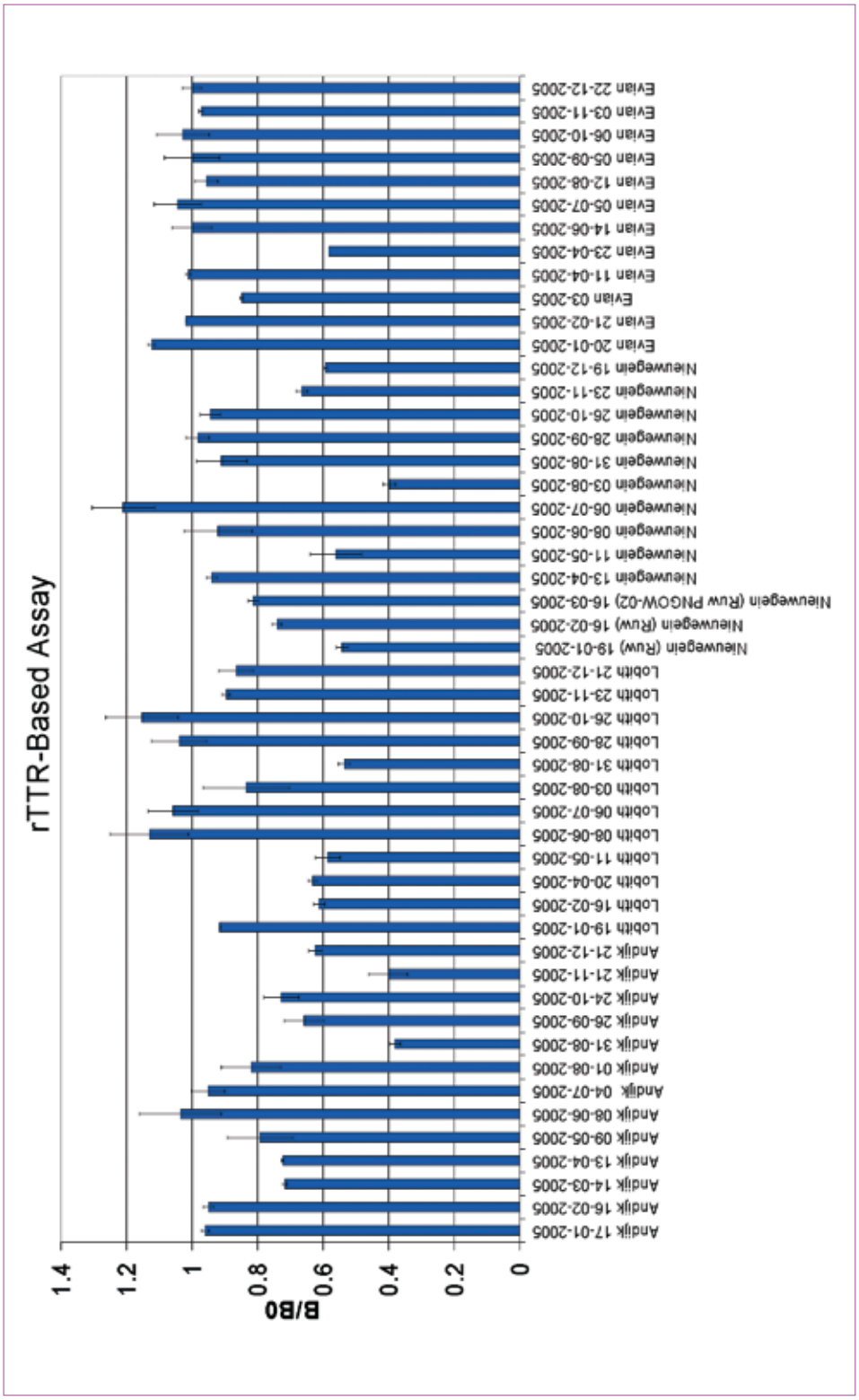

Figure a: B/B0 values found in the Biacore sensorassay using rTTR.

Overview of the results of the rTTR (Figure a) and TBG (Figure b) sensor assays, respectively, for all the samples. Data are expressed as B/Bo value.

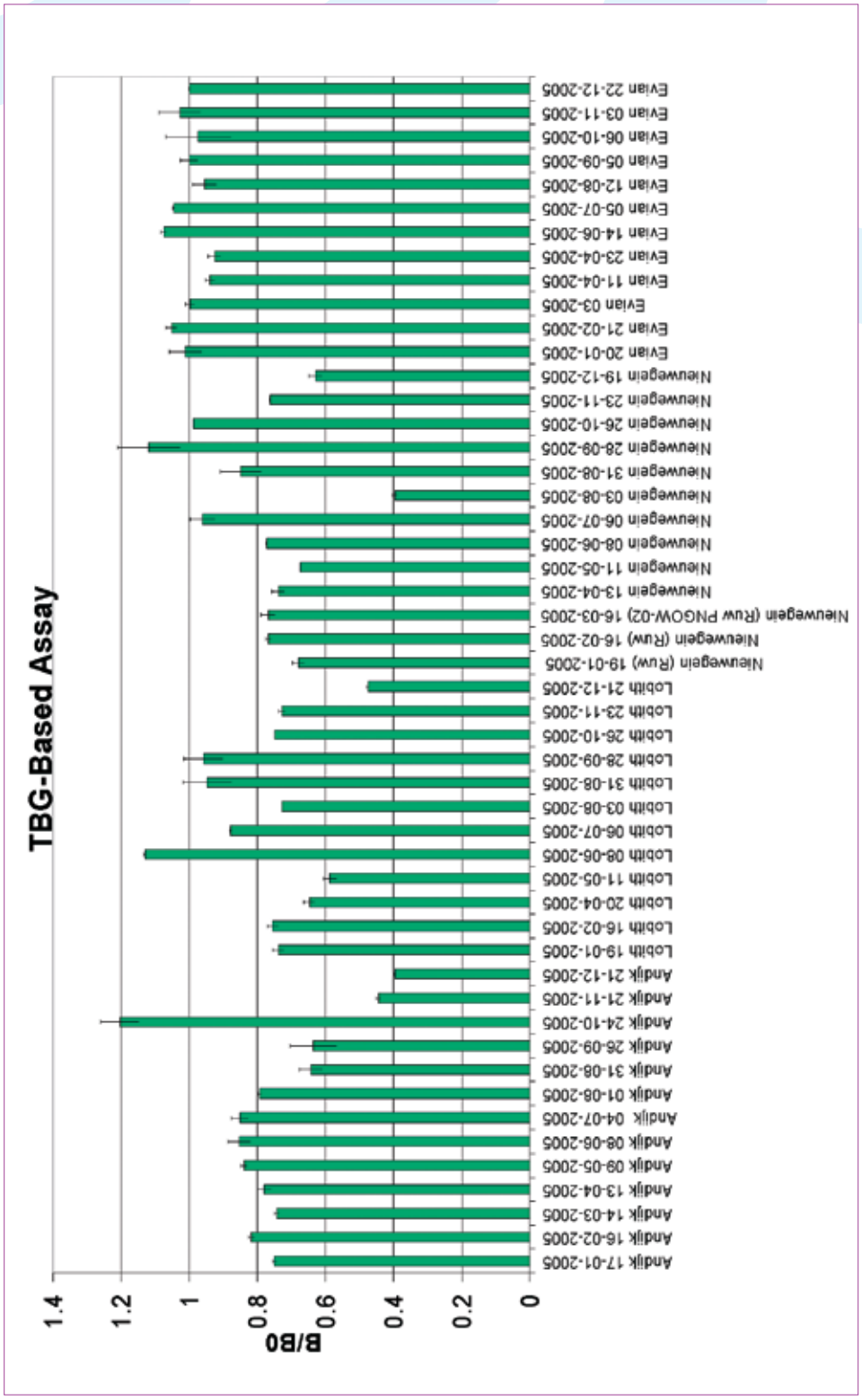

Figure b: B/B0 values found in the Biacore sensorassay using TBG.

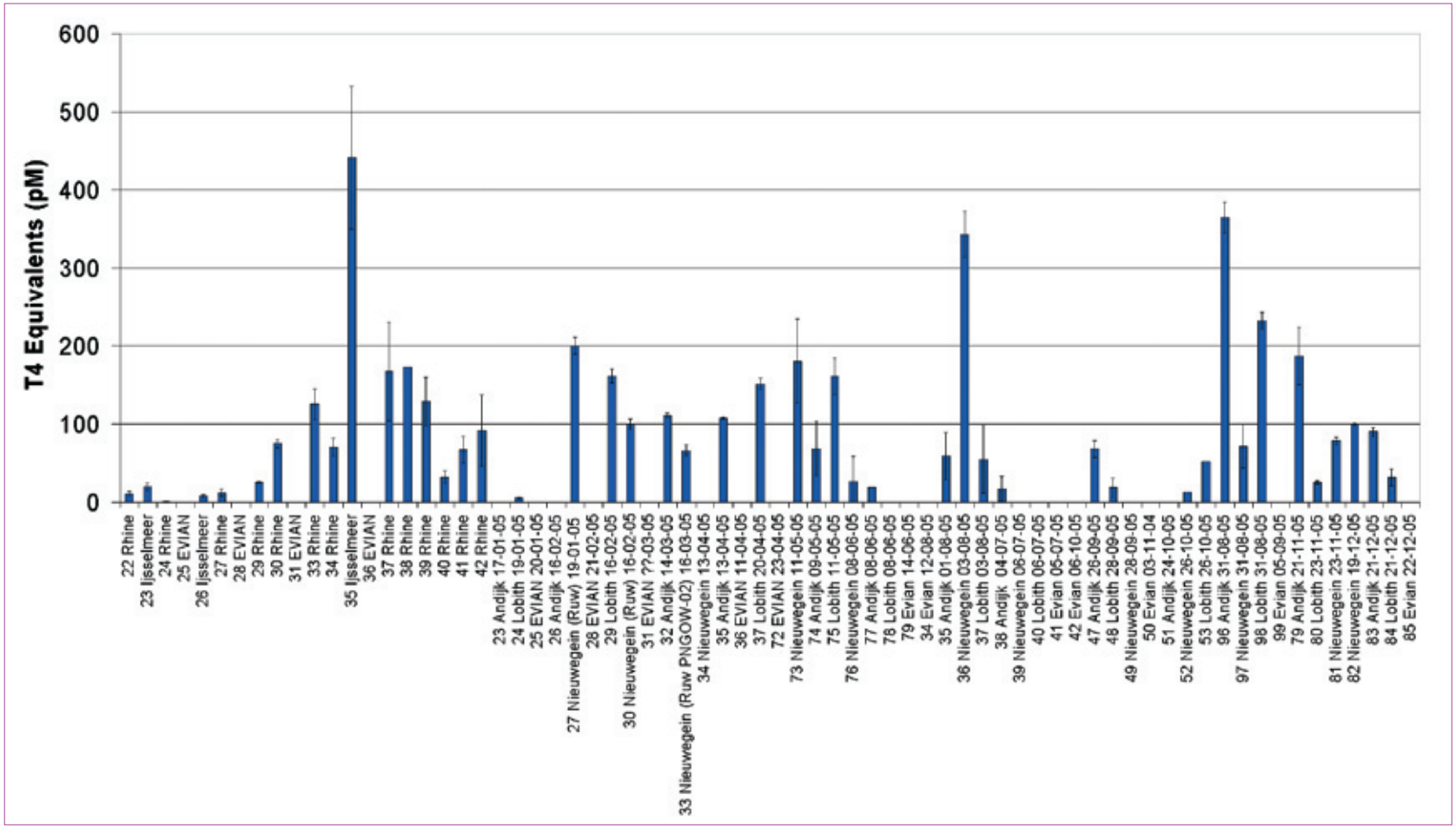

Figure c: Values of the samples expressed as T4 equivalents about background level in the TTR-assay.

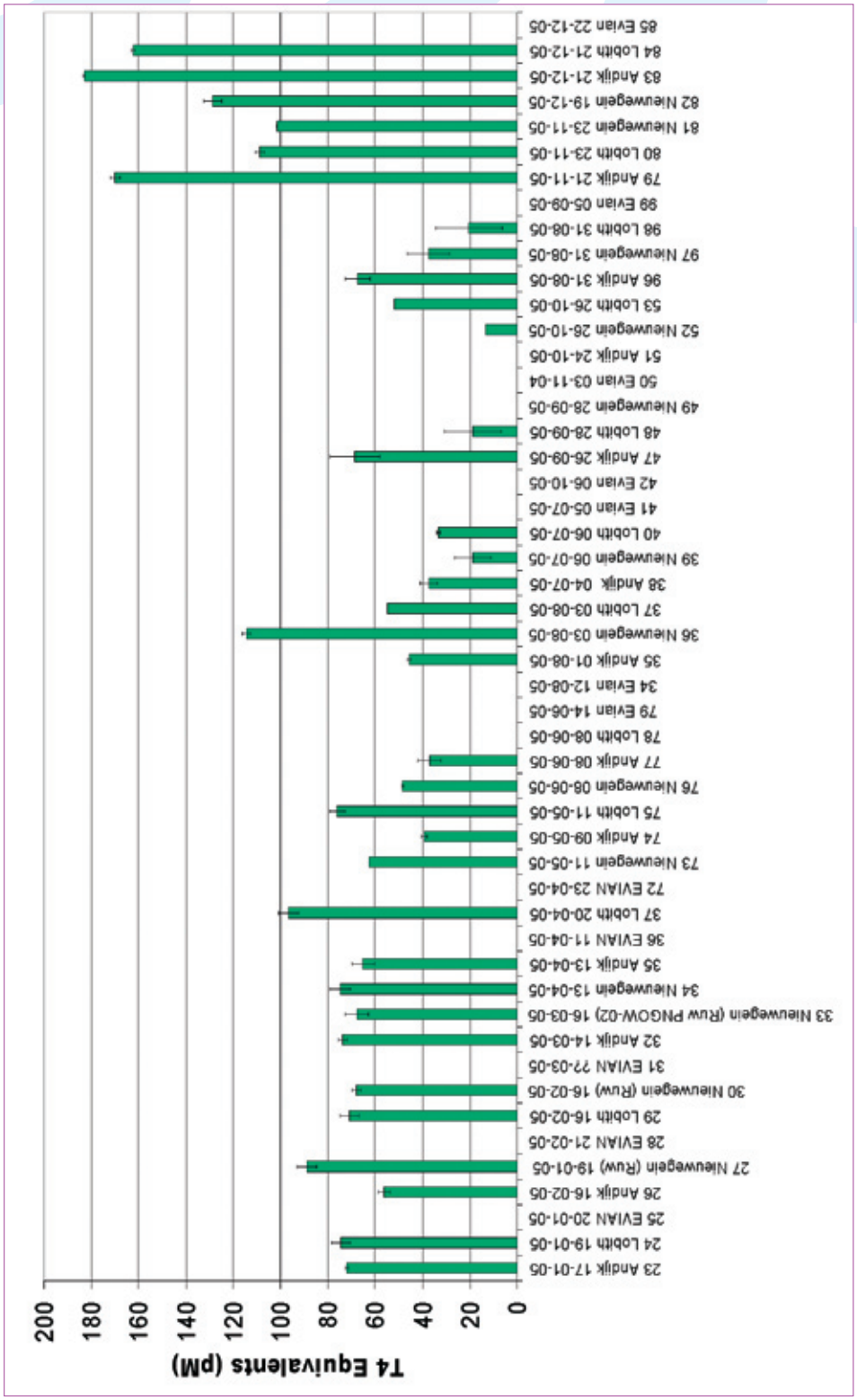

Figure d: Values of the samples expressed as T4 equivalents about background level in the TBG-assay.

Colofon

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