

Quality of Life and Management of Living Resources

Proposal Nr. QLRT-PL1999-01567

Sexual Identification and Development in the Swordfish – Improved Determination Tools for more Efficient Stock Assessment and Implementation of Control Measures

SIDS

SECOND PROGRESS REPORT

(Accepted)

1.1.1 - 5. Sustainable agriculture, fisheries and forestry and integrated development of rural areas including mountain areas

**1.1.1- 5.1. 2 .Sustainable fisheries and aquaculture
1.1.1- 5. 4. 3. Monitoring and enforcement of the CFP**

Project Progress Summary

Section 1: PROJECT IDENTIFICATION		NOT CONFIDENTIAL	
Information to be provided for project identification			
Title of the project: Sexual Identification and Development in the Swordfish – Improved Determination Tools for more Efficient Stock Assessment and Implementation of Control Measures			
Acronym of the project: SIDS			
Type of contract		RTD	Total project cost (in euro) 385,442 €
Contract number QLRT-PL1999-01567	Duration (in months) 24 Months		EU contribution (in euro) 344,465 €
Commencement date 01.03.2000		Period covered by the progress report (e.g. 1 February 2000 – 31 January 2001) 01.03.2000 – 01.03.2001	
PROJECT COORDINATOR			
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Key words (5 maximum - Please include specific keywords that best describe the project.). Swordfish, muscle biopsy, sex determination, steroids, vitellogenin			
World wide web address (the project's www address) http://www.uni-duesseldorf.de/WWW/MathNat/Zoophys/bridges/swordfish.htm			

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Objectives:

To sample swordfish populations from the eastern, central and western Mediterranean Seas and develop methods to determine steroid hormone concentrations and vitellogenin concentrations in plasma and tissue of these fish over a seasonal reproductive cycle. These will then be used to determine sex and sexual maturity and compared with histological studies made at the same time. Using this study as a calibration a muscle biopsy test will be evolved for standard determination of sex and maturity from single muscle samples. This will be tested by simulated market conditions and “double blind” experiments under field conditions.

The objectives will be carried out over two fishing seasons with the second season serving as market testing. In the first season the main objectives are to determine the stability of the sampling methods and to develop the necessary methods for determining steroids and vitellogenin in swordfish plasma and muscle.

Results and Milestones:

From the second fishing season (2001) 184 fish were sampled in Italy (62 Males and 122 Females), 184 fish were sampled in Greece (86 Males and 98 Females) . Data from Spain for the second progress report has not been supplied yet.

The histological analysis of testes led to the identification of a high percentage of intersex (25 %) showing the presence of isolated or grouped oocytes inside the testicular tissue of Spanish and Italian fish. No evidence was found in specimens taken from Greek waters.

All steroid tests have been successfully applied to both plasma and tissue from the fishing season 2001 where possible. Some problems did arise in the quantity and quality of the muscle samples supplied from the sampling groups.

The samples from 2000 were all assessed for Vtg in both plasma and tissue using the newly established ELISA test. Working with Standards produced excellent results, however on measuring plasma samples all fish were deemed to contain low but significant amounts of Vtg at around 1mg/ml. As this would be unusual in all fish the results were re-examined and especially the Vtg ELISA.

From these results it would appear that native Vtg is transported in the plasma with a secondary protein. This protein which appears to have the same MW as Vtg must first be removed before anti-body generation. The general trends will probably remain but the absolute levels of Vtg must be re-measured.

The earlier evidence of endocrine disruption shown in fishing season I have been confirmed from fish taken in fishing season II.

Benefits and Beneficiaries:

It would appear that one cannot extrapolate from the successful sex- determination methods in the BFT to the Swordfish. This however may hold only for Mediterranean Swordfish where endocrine may be apparent. The aim of the present study has not been achieved but the results indicate that urgent work is now necessary on Mediterranean swordfish populations to determine the extent and the consequences of “endocrine disruption” on the fishery structure and on its impact on human populations.

Future Actions (if applicable):

Generation of new Antibody for Swordfish Vtg is now required which will take approximately 90 days + measurement of samples 14 days.

Further assessment of pilot samples received from South Africa , Hawaii and possibly Reunion.

Prepare new proposals covering all aspects of the threat of “Endocrine Disruption” in large pelagic predators. Unfortunately in the latest round of awards the fisheries aspects of endocrine disruption have been seriously neglected.

Progress Report

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SIDS - SECOND PROGRESS REPORT- BARI

Sample Collection

During the period 2001-2002 the Research Unit of the University of Bari has been engaged in the completion of field sampling. Biological material (plasma, muscle, gonad and liver samples) was obtained, during the period March-December 2001, from 184 swordfish (62 males and 122 females) caught commercially by long lines and drift nets in the North Ionian Sea (Gulf of Taranto). All the specimens were measured to the nearest cm in lower jaw fork length (LJFL) and to the nearest 500 g in eviscerated body weight (W_B). Gonad weight (W_G) was measured to the nearest g. The gonadosomatic index (GSI) was calculated as $100 W_G/W_B$.

Blood was collected from the heart with heparinized syringes and cannula. Syringes, cannula and were been rinsed in advance with a solution containing 200 mM NaCl; 8×6 KCl, 8000 I.U. ml⁻¹ Sodium heparin and 1 mM phenylmethylsulphonylfluoride (PMSF, Sigma), pH 7×3. Blood was kept on ice after sampling at sea and then centrifuged at 5000 x g for 15 min. Plasma was collected using a plastic pipette and stored at -20°C in the laboratory.

Muscle samples were taken from the ventral abdominal wall and kept on ice after sampling at sea and then stored at -20°C.

Fragments of ovaries and liver were taken soon after capture and immediately fixed in Bouin's solution or formaline.

Laboratory work

The Research Unit of the University of Bari is responsible for the histological, histochemical and immunohistochemical analyses of swordfish gonads and liver collected in Italian, Greek and Spanish seas.

As decided during the second meeting hold in Athens in January 2001 this Research Unit has taken in charge the processing of gonad and liver samples collected in Italian and Spanish seas. The Research Unit of the University of Athens has taken in charge the processing of gonad and liver samples collected in Greek seas.

Due to the subjectivity of the classification of gonad maturity stage, an inter-calibration session between the Research Units of the University of Bari and Athens has been planned in order to avoid differences in the interpretation of the histological features.

Histological and Histochemical Methods

All the samples collected in Italy (182) and Spain (59) have been processed and analysed.

Gonad samples previously fixed in Bouin's solution or formaline were dehydrated in increasing ethanol concentrations, clarified in Histolemon and embedded in paraffin wax. Sections (5 µm thick) were stained with Haematoxylin – Eosin, Azan, or Mallory's trichrome.

Histochemical detection of carbohydrates was performed on paraffin sections using the periodic acid – Schiff reaction (PAS) and Alcian blue pH 2.5.

Oocyte diameters were measured on histological slides using Quantimet (Leica, Cambridge, UK) image analyser.

Immunohistochemical Detection of VTG-Like Material.

The immunohistochemical detection of vitellogenin (VTG) like material was performed on ovary and liver sections using rabbit anti swordfish VTG serum (abSwo-VTG) obtained by the Research Unit of the University of Duesseldorf. The sections were deparaffinized, hydrated and pre-treated for 30 min with 0.3% H₂O₂ in methanol to inhibit endogenous peroxidase activity. They were then incubated for 30 min in normal horse serum (NHS) diluted 1:10 in phosphate buffered saline (PBS) (0.01 M phosphate buffer pH 7.4 containing 0.15 M NaCl) to block non-specific binding sites for immunoglobulins. The sections were then incubated for 1 h at 37 °C in a moist chamber with the abSwo-VTG diluted 1:10000 in PBS containing 0.1% BSA. After rinsing for 10 min in PBS, immunohistochemical visualisation was obtained using the Vectastain Universal Elite Kit (Vector, Burlingame, CA). Peroxidase activity was visualised by incubating for 10 min with Vector DAB Peroxidase Substrate Kit (Vector, Burlingame, CA), which produces a brown precipitate.

Results

All the Italian and Spanish samples collected during the second fishing season (2001) have been analysed. Gonad samples have been classified according to the histological scale set up during the first year of the research project. The intercalibration session has been carried out in April 2002 in order to standardize the classification of gonads between this Research Unit and the Athens one. Seasonal changes of hepatic synthesis of vitellogenin have been analysed in adult females throughout the reproductive cycle and correlated with ovarian maturity stage.

The histological analysis of testes led to the identification of a high percentage of intersex (25 %) showing the presence of isolated or grouped oocytes inside the testicular tissue.

The liver of male specimens showed the presence of anti vitellogenin positive cells indicating that Mediterranean swordfish could be exposed to estrogen-mimicking substances.

SIDS - SECOND PROGRESS REPORT- ATHENS

Sampling

Swordfish blood sampling in Greece during 2001, was carried out on four (4) professional fishing boats using the standard or American type swordfish long line.

Their home port was Kastelli in Crete island (S. Aegean Sea) and their activities covered S. Aegean Sea and Levantine basin areas.

Fishing operations took place usually more than 10 miles from coast, a high seas activity. Sampling covered the period from March to September. Swordfish fishing period in Greece starts in February and ends in September. No samplings during February were possible due to few exits of the boats (non favorable meteorological conditions).

During sampling, various non-biological data were recorded:

- Location (GPS)
- Sea surface temperature (SST in Celsius degrees)
- Setting time (start – end)
- Retrieving time (start – end)
- Type of gear (No of hooks, Bait, Full length of long line, Fishing depth of long line)

Biological data and samples concerned:

- Lower Jaw Fork Length (LJFL in cm)
- Gilled & Gutted Weight (GWT in kg)
- Sex identification (macroscopic)
- Gonads weight (left and right separately)
- Gonads length (left and right separately)
- Gonad stage estimation (macroscopic)
- Calculation of GSI
- Blood samples
- Muscle samples
- Liver samples
- Gonad samples

Histology of the gonads

A total of 184 swordfish were sexed on board by macroscopic examination of the gonads and the maturity stage was recorded. Histological sections of the gonads were prepared for 172 swordfish. Of them, 83 samples were identified for sex and maturity stage in the lab of the University of Bari.

Samples sent to :

- BARI - October 2001, 79 samples of liver tissue in formaline
- DUESSELDORF – June 2001,
 - 82 plasma and 114 muscle for Duesseldorf Laboratory
 - 67 plasma and 114 muscle for Sheffield Laboratory
- DUESSELDORF – October 2001,
 - 70 plasma and 70 muscle (+16 market simulation) for Duesseldorf Lab
 - 64 plasma and 70 muscle for Sheffield Laboratory

Detailed data collection concerning 2001 is presented in the tables below

Table I. Biological data and samples collection for year 2001

General Data	
Sampling Days	49
Sampling Months	7
Sampled Fish	185

Blood sampling Data	
	No of fish
Total	152
Whole blood	2
Emergency serum (no centrifugation)	14
Serum	136

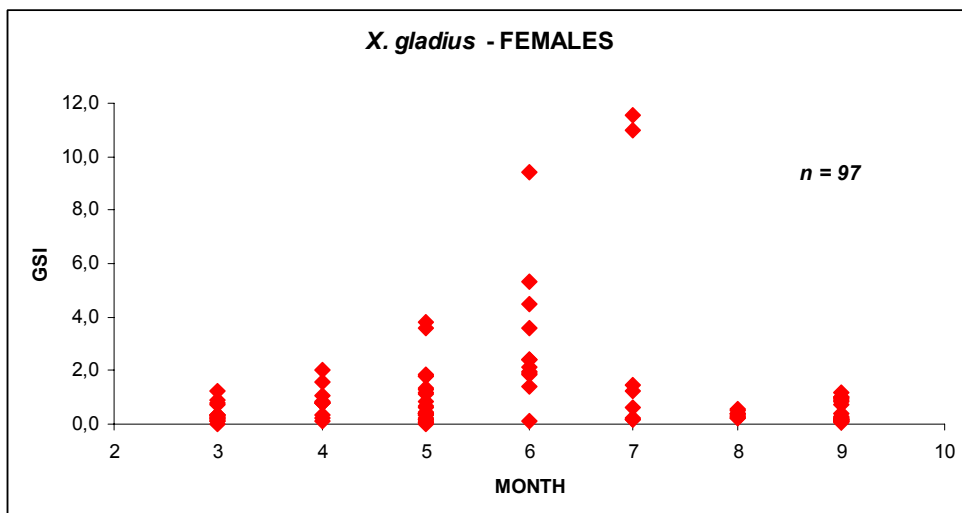
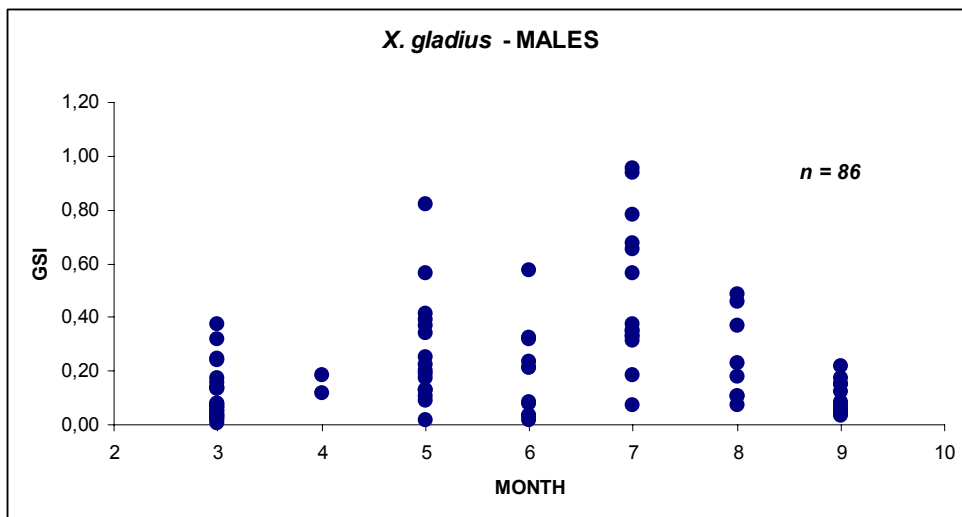
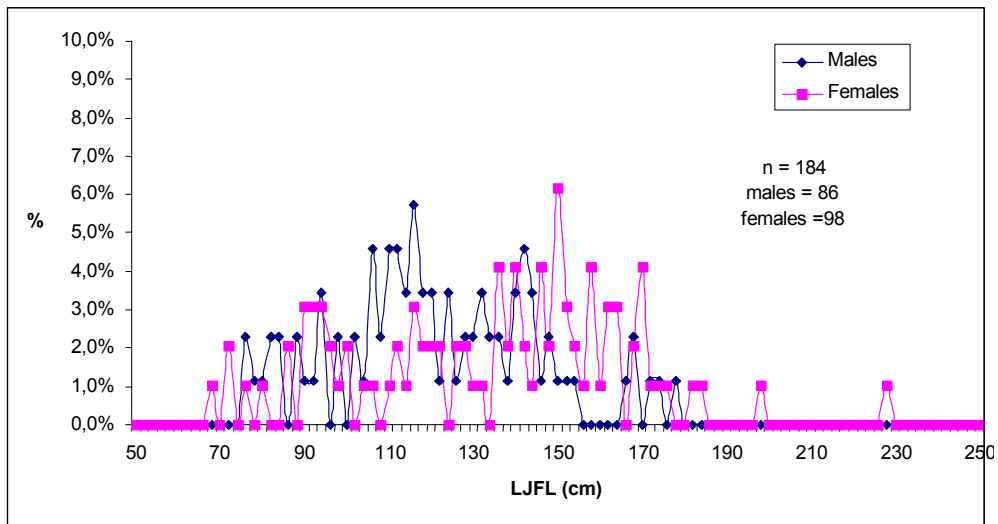
Gonads Data	
	No of fish
Total	184
Gonads weight	184
Gonades in formaline	181
Gonades frozen sample	181

Other Tissue Data	
	No of fish
Muscle frozen	184
Liver frozen	131
Liver in formaline	0

Sex and Maturity Data	
	No of fish
Sex (macroscopic)	184
Sex (microscopic)	172
Gonad stage (macroscopic)	184
Gonad stage (microscopic)	159
GSI	183
GSI Male	86
GSI Female	97

Table II. Distribution of samples by month in 2001

Month	Males	Females	Total
March	23	14	37
April	2	10	12
May	16	26	42
June	11	12	23
July	14	8	22
August	8	8	16
September	12	20	32
Total	86	98	184



SIDS – SECOND PROGRESS REPORT – SHEFFIELD

1. Steroid analysis for 2001 sampling.

Steroid analysis was performed on plasmas of 81 swordfish and 63 muscle specimens from the 2001 sampling season. As with the 2000 season sampling, muscle tissue was very fatty and insufficient good quality fat free tissue was found in many samples. Blood sampling also gave problems in that whole blood samples were obtained rather than plasma and could not be analysed. It is apparent that sampling fish during a commercial fishing operation far from a home port is not conducive to good sampling, and that, not surprisingly, the quality of samples obtained is much poorer than that expected from a normal laboratory sampling.

2. Steroid data analysis from the 2000 and 2001 sampling

Regression analysis of samples for both 2000 and 2001 showed no correlation between plasma and muscle levels. There was also no correlation between muscle steroid concentrations and either sex or maturational status for samples from 2000. Although the few previous studies on tissue concentrations suggest levels approx 1/10 of those in plasma our values for muscle were, in general, comparable in level to those of plasma. This together with the large amount of fat in the muscle samples suggests that the fatty nature of swordfish muscle interferes with the ELISA. Although tedious and time-consuming methods are available to remove such fat this would defeat the objective of producing a simple and rapid analytical method.

The complete dataset for 2000, including samples not available when the First Periodic Report was submitted, has now been replotted to show relations between steroid levels, season, sex and sexual maturity. The 2001 dataset is awaiting histological sex and maturity data, but on the macroscopic data presently available confirms the results obtained in 2000. This suggests that while plasma estrogen and 11-ketotestosterone are good indicators of sexually mature female or male fish respectively, there are a few fish (usually with low GSI) which do not conform to the normal pattern. Estrogen tends to be high in males when GSI is low at Stages 5 and 3', while in females high KT is most apparent at Stages 1 and 5 when again the gonads are regressed. The data indicate that plasma steroid hormone measurement cannot be used as a reliable indicator of sex in swordfish.

SECOND PROGRESS REPORT – DÜSSELDORF

Development of Swordfish Vtg ELISA

Using the Vtg antibody which was obtained as detailed in the first report the samples of plasma and muscle from 2000 were all measured given average results for plasma as shown below (Fig 1).

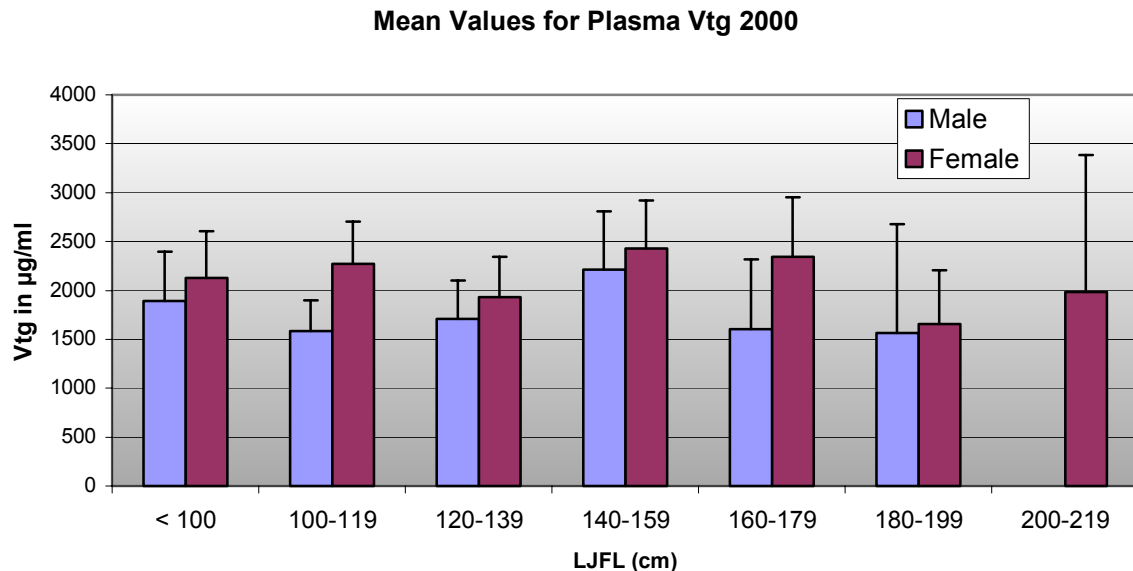


Figure 1 ELISA results for Plasma samples from 2000 plotted against Lower Jaw Fork Length

These results were unsatisfactory and it was noted that although the standard functioned perfectly well however when the standard samples were spiked with plasma an inhibition occurred. On carrying out further studies using different ion-chromatography conditions available with the FPLC the Vtg peak could be separated from a second peak. Under gelfiltration conditions it was found that both peaks from the ion-chromatography had the same Molecular Weight. On re-chromatographing the standard the retention time on the ion chromatography exchanger was reduced compared to the original plasma samples and two peaks were seen (Fig.2). On collecting Peaks 1 and 2 and testing them with the ELISA only peak 1 showed a response. Fig.3. These results indicate that the ELISA antibody is not specific for Vtg but also for a transport protein. Comparisons made with Vtg from the BFT did not show the same properties although an aggregation of proteins appears also to occur. A new more specific antibody must first be generated before absolute values can be measured. However the major finding that Vtg is present in male fish can also be confirmed by the ion-chromatography experiments.

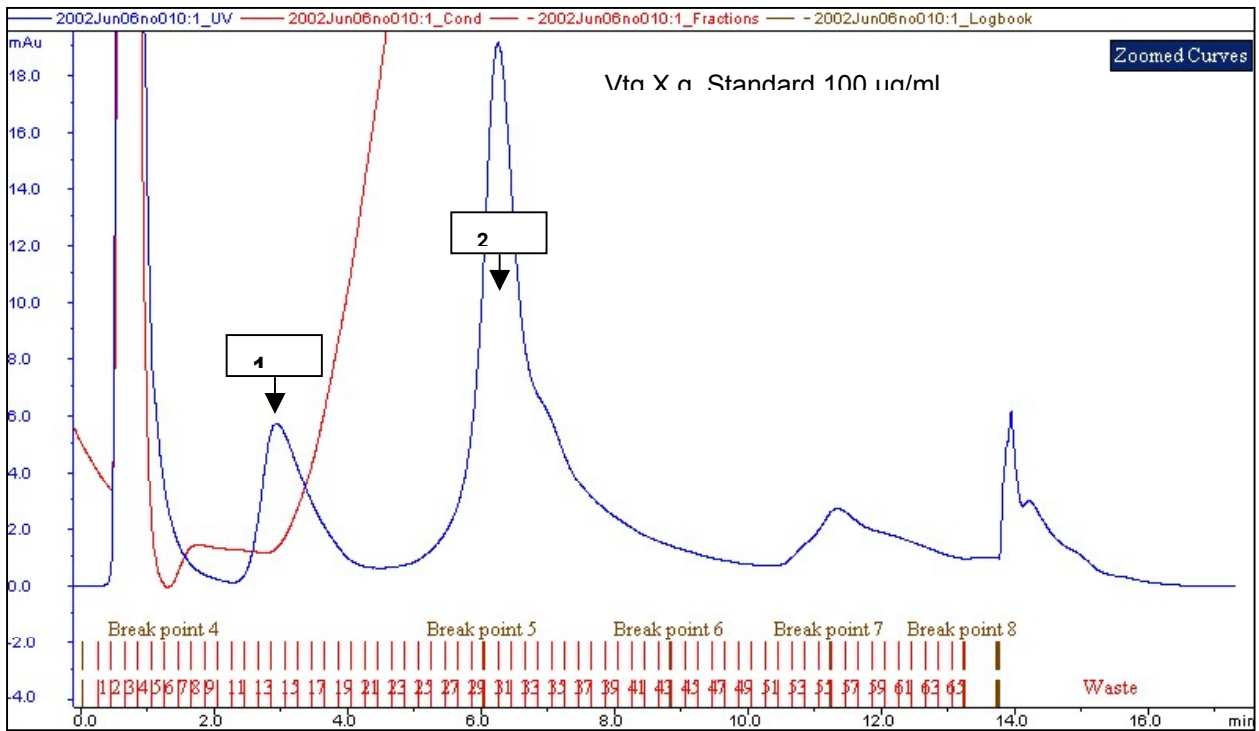


Figure 2. Details of the ion exchange chromatography for the Vtg Standard.

ELISA - Ion-Chromatog. Standards and Samples

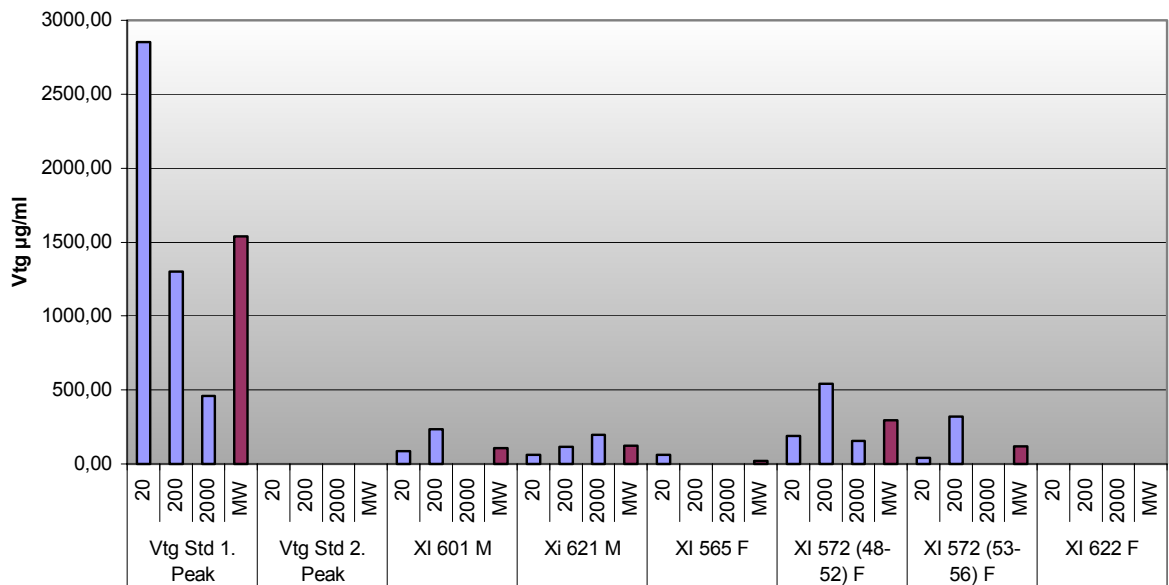


Figure 3. Details of the results from the ELISA test on the isolated peaks taken from the ion- chromatography and also plasma samples.