EROD (7-ethoxyresorufin O-deethylase) and protein intercalibration exercise with homogenised fish liver samples

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Contents

Participating laboratories

There were 10 participating laboratories, which submitted ethoxyresorufin-O-deethylase (EROD) and protein results from all prepared microsomal and S9 fractions from fish liver samples.

Exposure study

The exposure study was performed at the Dept. of Biological and Environmental Sciences, University of Gothenburg, in October 2022. Rainbow trout (*Oncorhynchus mykiss*) were exposed to a low and high combined dose of beta-naphthoflavone (0.1 and 10 mg/kg) and pyrene (0.01 and 0.1 mg/kg) through intraperitoneal injection (IP) for five days as well as a control group (Figure 1). The chemicals were dissolved in peanut oil and peanut oil was also used as a carrier control in the control group. Five fish were used per treatment, resulting in 15 fish in total.

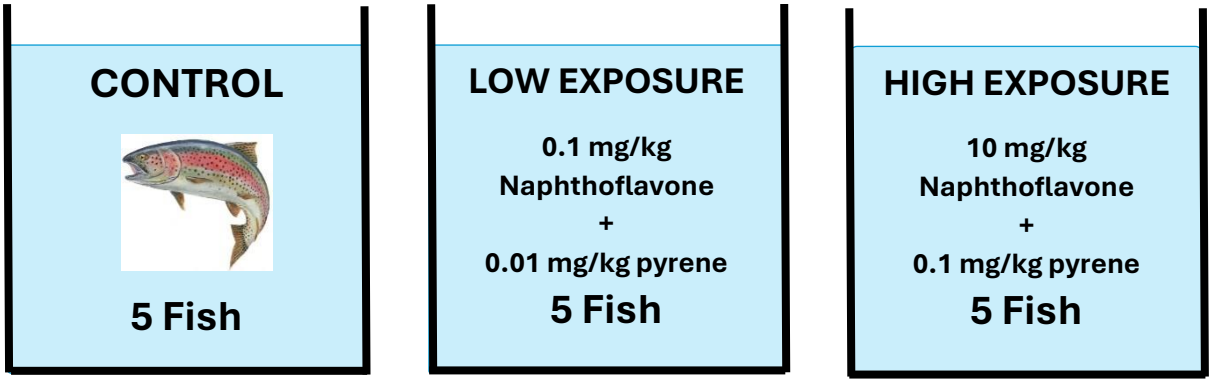

Figure 1. Exposure of rainbow trout to low and high combined doses of naphthoflavone and pyrene.

Following the five-day exposure period, the fish were euthanised, and the livers excised from the 15 fish. The microsomal and S9 fractions of fish liver samples were prepared and distributed to all participating laboratories. Livers were homogenized (3 x 3 strokes with glass / Teflon) in four volumes (w/v) of homogenisation buffer (Na^+/K^+-phosphate buffer (pH 7.4) containing 0.15 M KCl). The homogenates were centrifuged at 10,000 g for 20 min in order to obtain the S9 fraction. Half of the supernatant was re-centrifuged at 105,000 g for 60 min. The 105,000 g pellets were resuspended in homogenization buffer containing 20% glycerol in order to obtain the microsomal fraction. All preparatory steps were carried out at 0–4°C. Both S9 and microsomal fractions were mixed thoroughly to obtain an homogenous sample and divided into 15 aliquots. The aliquots were frozen and stored in -80°C until analysed.

Each participating laboratory was sent 30 samples consisting of 15 microsomal fractions and 15 S9 fractions. The samples represented the 3 treatment groups (control, low dose and high dose) with 5 replicate samples per treatment group.

Shipment of samples

All samples were distributed to each participating laboratory by courier on dry ice in thermally insulated polystyrene boxes. The delivery time varied between laboratory but in all cases the samples, when received, were reported to contain suitable amounts of dry ice remaining to maintain adequate cryo-storage. The samples were stored by each laboratory at -80°C prior to use.

Table 1. Summary of the samples sent to the participating laboratories.

Sample fraction	Control	Low exposure	High exposure	Total
S9	5	5	5	15
Microsomal	5	5	5	15
Subtotal	10	10	10	30

EROD and protein measurements

All samples were analysed for EROD and protein by the laboratory's method of choice. For EROD measurements, participating laboratories were recommended to use the ICES TIMES 23 protocol based on Stagg and McIntosh, 1998, although ICES TIMES 13 and 57 were also available as alternative approaches for EROD.

For protein measurements, laboratories were also asked to use their method of choice. However, it was recommended that protein measurements based on the Lowry colorimetric method should be used, with either bovine serum albumin (BSA) or bovine gamma globulin (BGG) as the protein standard. Participants were advised not to use the combination of Bradford protein assay and BSA standard.

Results and Discussion

Each laboratory was given a lab code, enabling the participating laboratories to be kept anonymous. The results are presented as the reported values from each laboratory (Table 2, Figure 2).

The ten laboratories reported the EROD activity and protein concentrations. Seven laboratories reported EROD activity in pmol/ min/ mg protein, whilst three laboratories reported EROD activity in nmol/ min/ mg protein. Initial attempts were made to convert the nmol results into pmol, but it was clear that the data was not comparable. Therefore, the results are present and discussed separately.

For the seven laboratories that reported EROD activity in pmol/ min/ mg protein, a reasonable agreement between the reported values for the different samples was shown. Although higher values were consistently reported by lab code 2, all the seven laboratories were able to differentiate well between the control, low and high exposure groups. These laboratories also consistently reported high EROD activity in the microsomal fractions compared to the S9 fractions.

For the three laboratories that reported values in nmol/ min/ mg protein (lab codes 4, 6 and 11), lab code 4 was not able to differentiate between the treatment groups and reported relatively low EROD activity for all samples. Lab code 4 also showed similar EROD activities for both the microsomal and S9 fractions. In contrast, Lab codes 6 and 11 were able to differentiate between the exposures, particularly between the control and high exposure treatments. Additionally, lab codes 6 and 11 reported higher EROD activity in microsomal fractions compared to S9 samples.

Table 2. EROD activity reported by the 10 laboratories for both the microsomal and S9 fractions for all 15 samples. Note that 3 laboratories reported values in nmol/min/mg protein and 7 laboratories reported values in pmol/ min/ mg protein.

A) microsomal fraction

Sample	Treatment	pmol/min/mg protein							nmol/min/mg protein		
		LAB CODE									
		1	2	3	5	8	9	10	4	6	11
1	low	763.76	2571.57	216.69	210.14	430.00	312.75	2247.90	9.16	9.67	15.87
2	low	1273.13	4902.23	361.43	349.62	688.90	527.62	1316.50	11.31	17.92	18.01
3	low	636.86	2014.66	178.52	138.88	378.70	266.67	1765.30	10.50	11.45	14.50
4	low	120.74	295.22	51.33	28.97	40.50	33.70	411.00	10.70	0.53	10.10
5	low	734.98	2046.84	179.98	164.07	331.40	245.27	2621.50	10.91	5.46	12.50
6	control	3.27	15.60	3.59	0.00	1.10	3.00	64.00	7.70	0.00	9.21
7	control	3.76	19.90	4.58	0.07	1.50	6.19	78.60	12.58	0.00	9.50
8	control	33.69	160.13	26.99	8.81	19.10	3.67	86.70	10.17	0.00	9.36
9	control	17.19	112.80	25.32	5.51	18.00	12.22	224.90	8.75		9.61
10	control	5.56	19.00	3.66	0.00	1.40	2.82	42.90	11.03	0.00	9.67
11	high	1808.56	8350.78	917.75	458.97	1472.50	603.89	1817.80	8.18	47.45	22.75
12	high	2791.35	11569.07	1013.89	1114.46	2012.60	963.42	683.20	6.30	50.68	33.69
13	high	2604.57	12984.04	1009.79	421.87	2212.50	885.95	615.40	11.35	61.78	32.46
14	high	3146.32	12995.49	1330.15	936.86	2120.80	1859.76	698.50	7.19	60.92	31.66
15	high	2837.92	13330.64	1233.71	317.99	2028.70	1006.88	340.60	7.89	67.30	35.46

B) S9 fraction

Sample	Treatment	pmol/min/mg protein							nmol/min/mg protein		
		LAB CODE									
		1	2	3	5	8	9	10	4	6	11
16	low	275.06	598.11	119.42	67.73	42.10	153.31	383.10	14.05	1.36	10.17
17	low	431.29	1130.80	132.10	88.55	53.30	230.39	508.10	9.88	2.60	10.81
18	low	215.42	437.50	67.07	35.92	23.60	102.38	292.10	17.73	1.31	8.64
19	low	7.52	28.85	12.94	2.02	0.90	13.22	50.40	15.60	0.00	6.26
20	low	198.17	332.18	64.33	31.80	24.60	90.48	322.30	13.70	0.00	7.83
21	control	1.10	2.47	0.00	0.00	0.10	0.00	28.50	12.23	0.00	7.20
22	control	2.81	5.03	2.94	0.00	0.20	0.00	43.70	11.19	0.00	7.08
23	control	6.33	36.58	6.34	0.14	1.70	5.48	30.30	11.62	0.00	6.81
24	control	5.47	32.55	6.05	0.00	1.10	6.23	62.30	10.22	0.00	6.64
25	control	2.64	2.39	2.96	0.00	0.10	0.00	0.00	22.47	0.00	6.13
26	high	612.37	1665.22	205.69	245.40	115.20	278.63	939.80	9.73	5.62	15.84
27	high	800.00	2201.49	142.40	224.43	146.50	338.78	1683.60	10.10	5.32	22.28
28	high	717.36	1759.09	212.16	233.11	128.00	378.28	724.20	12.09	6.94	20.24
29	high	1922.71	2712.29	281.71	262.43	162.20	433.45	966.60	12.25	5.82	18.01
30	high	884.82	2356.02	304.43	188.92	135.80	421.20	1014.90	8.31	5.21	20.59

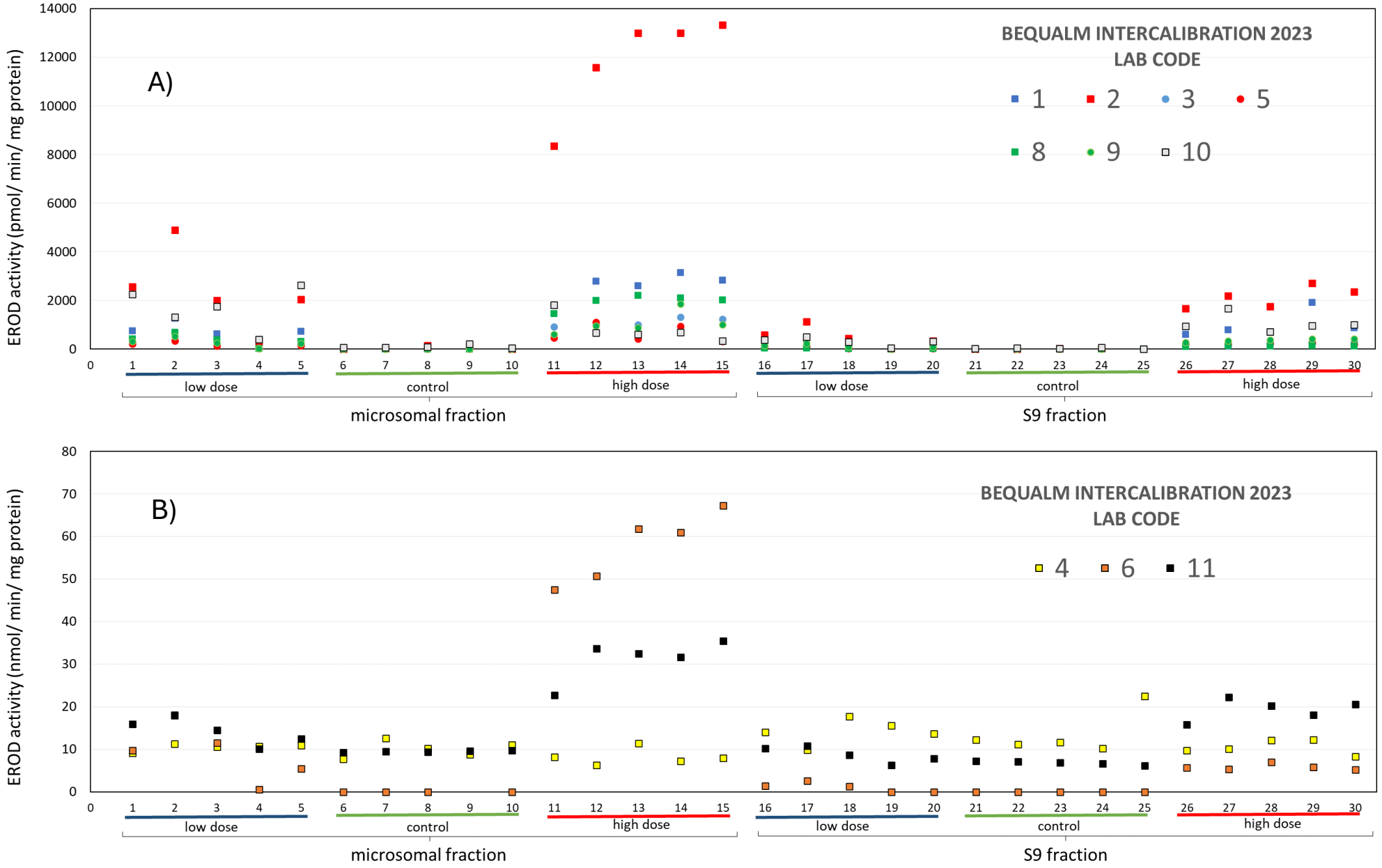

Figure 2. EROD activity in liver microsomal and S9 fractions reported in A) pmol concentrations by 7 laboratories and B) nmol concentrations by 3 laboratories.

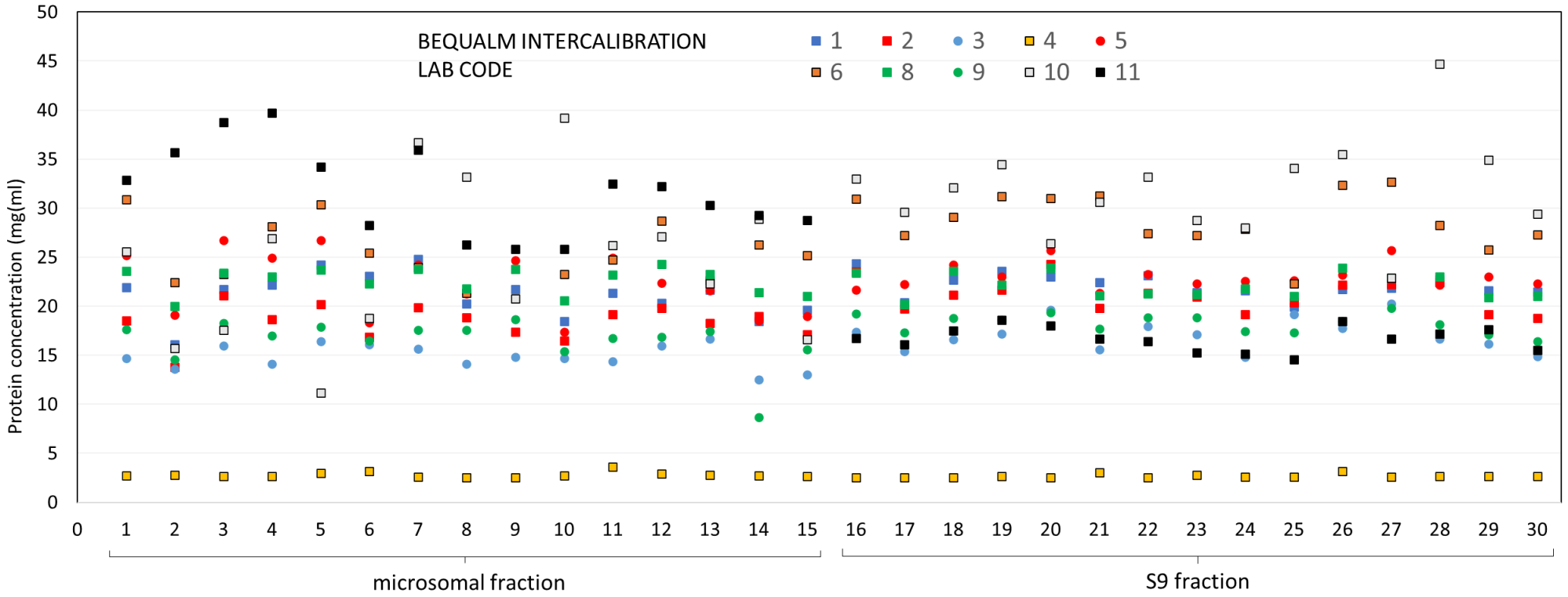

Figure 3. Protein concentrations in liver microsomal and S9 fractions as reported by the 10 participating laboratories.

The protein concentrations reported by all 10 laboratories for all microsomal and S9 fractions are shown in figure 3. Consistently low protein concentrations were reported by lab code 4 for all microsomal and S9 samples and were markedly lower than all other laboratories. When excluding lab code 4, the majority of protein concentrations lay within 15 to 30 mg/ ml with no obvious difference between microsomal and S9 fractions. Only lab code 11, reported consistently higher concentrations of protein in the microsomal fractions compared to the S9 fractions.

Detection of outliers

Suspect outliers for each sample were identified using the Dixon Q-test, a simple test that enables the detection of outliers to be perform in a statistically robust manner. Using ranked values for each sample separately, the experimental Q-value (Q_{exp}) was calculated using the formula:

$$Q_{exp} = (X_N - X_{N-1}) / (X_N - X_1)$$

Where X_1 is the lowest value and X_N is the highest ranked sample measurements. The obtained Q_{exp} was compared to a critical Q-value (Q_{crit}) at 95% confidence limits. In cases where the Qexp was greater than Q_{crit} the extreme measurement for the particular sample was identified as an outlier. The Dixon Q-test was only performed on the laboratory values that report EROD activity in pmol/ min/ mg protein (Table 3). This included 7 laboratories in total. The 3 laboratories that reported in nmol/ min/ mg protein were not included, since the values, even after conversion, where not comparable.

Table 3. Results of the Dixon Q test indicating the extreme outliers for EROD activity from the individual samples.

	Samples (microsomal fraction)														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Qexp	0.137	0.788	0.133	0.303	0.234	0.756	0.747	0.469	0.511	0.557	0.828	0.806	0.826	0.801	0.806
Qcrit	0.568	0.568	0.568	0.568	0.568	0.568	0.568	0.568	0.568	0.568	0.568	0.568	0.568	0.568	0.568
Outlier	No	Yes	No	No	No	Yes	Yes	No	No	No	Yes	Yes	Yes	Yes	Yes

	Samples (S9 fraction)														
	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Qexp	0.387	0.578	0.351	0.435	0.032	0.913	0.885	0.172	0.478	0.107	0.468	0.252	0.634	0.310	0.604
Qcrit	0.568	0.568	0.568	0.568	0.568	0.568	0.568	0.568	0.568	0.568	0.568	0.568	0.568	0.568	0.568
Outlier	No	Yes	No	No	No	Yes	Yes	No	No	No	No	No	Yes	No	Yes

The Dixon Q test detected 8 outliers for the microsomal fraction samples, including sample 2, 6, 7, 11-15. Five additional outliers were also identified for the S9 fraction (17, 21, 22, 28 and 30). These outliers were excluded from the Z score calculation.

For the microsomal samples 6 and 7 and the corresponding S9 fraction samples 21 and 22, lab code 10 were responsible for the outlier values. For all other outliers, including microsomal samples 2, 11 to 15 and S9 fractions 17, 28 and 30, lab code 2 was responsible for the outlier values. The higher EROD activities of the microsomal fractions compared to the S9 fractions may partly explain why more outliers were found in former than the latter.

The Dixon Q test was also performed on the protein concentrations for each microsomal and S9 fraction (Table 4). In this case, the outliers identified represented the lower protein concentrations reported by lab code 4, where outliers were identified in 3 of the microsomal samples (6, 9, 13) and 7 of the S9 samples (16-18, 20, 23, 24, 27). These protein outliers were removed from the z score calculations.

Table 4. Results of the Dixon Q test indicating the extreme outliers for protein concentrations from the individual samples. Note, n=9 for sample 9 with a Q_{crit} of 0.493 (95% CL), n =10 for all other samples.

	Samples (microsomal fraction)														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Qexp	0.397	0.329	0.370	0.310	0.263	0.515	0.383	0.377	0.528	0.328	0.373	0.445	0.503	0.224	0.397
Qcrit (95% CL)	0.466	0.466	0.466	0.466	0.466	0.466	0.466	0.466	0.493	0.466	0.466	0.466	0.466	0.466	0.466
Outlier	no	no	no	no	no	yes	no	no	no	no	no	no	no	no	no

	Samples (S9 fraction)														
	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Qexp	0.467	0.476	0.476	0.457	0.543	0.444	0.452	0.479	0.480	0.381	0.451	0.468	0.334	0.418	0.458
Qcrit (95% CL)	0.466	0.466	0.466	0.466	0.466	0.466	0.466	0.466	0.466	0.466	0.466	0.466	0.466	0.466	0.466
Outlier	Yes	Yes	Yes	no	Yes	no	no	Yes	Yes	no	no	Yes	no	no	no

Calculation of z scores

Individual z scores were calculated using the following formula:

$$Z\ score = \frac{(measured\ value - mean\ value)}{standard\ deviation\ from\ all\ laboratories}$$

An assessment criterion for each Z score was perform based on the ISO43 guideline.

Z score < 2	Satisfactory
2 < Z score < 3	Questionable
Z score > 3	Unsatisfactory
Z score > 6	Extreme

Table 5. Calculated z scores for the microsomal (1-15) and S9 (16-30) fractions for the 7 laboratories that reported EROD measurements in pmol/ min/ mg protein. Outliers based on the Dixon Q test were removed from the z score calculation (blank cells). Z scores above the threshold of 2 indicating questionable results are highlighted in yellow.

Sample	Lab code						
	1	2	3	5	8	9	10
1	-0.20	1.59	-0.74	-0.75	-0.53	-0.65	1.27
2	1.19		-0.89	-0.92	-0.15	-0.51	1.29
3	-0.17	1.58	-0.75	-0.80	-0.50	-0.64	1.27
4	-0.13	1.02	-0.58	-0.73	-0.65	-0.70	1.78
5	-0.17	1.13	-0.72	-0.73	-0.57	-0.65	1.70
6	-0.20	1.98	-0.15	-0.78	-0.59	-0.25	
7	-0.31	1.94	-0.20	-0.83	-0.63	0.03	
8	-0.26	1.98	-0.38	-0.70	-0.52	-0.79	0.68
9	-0.52	0.65	-0.42	-0.66	-0.51	-0.58	2.03
10	-0.34	0.53	-0.46	-0.69	-0.60	-0.51	2.07
11	1.05		-0.44	-1.20	0.49	-0.96	1.06
12	1.69		-0.52	-0.39	0.72	-0.58	-0.93
13	1.46		-0.31	-0.97	1.03	-0.45	-0.75
14	1.63		-0.39	-0.83	0.49	0.20	-1.10
15	1.56		-0.06	-0.99	0.74	-0.29	-0.97
16	0.20	1.82	-0.57	-0.83	-0.96	-0.40	0.74
17	1.01		-0.58	-0.81	-0.99	-0.05	1.42
18	0.31	1.75	-0.65	-0.85	-0.93	-0.42	0.81
19	-0.51	0.70	-0.21	-0.83	-0.89	-0.19	1.92
20	0.35	1.36	-0.66	-0.91	-0.96	-0.46	1.28
21	0.49	1.84	-0.61	-0.61	-0.51	-0.61	
22	0.47	1.53	0.53	-0.88	-0.78	-0.88	
23	-0.41	1.65	-0.41	-0.84	-0.73	-0.47	1.22
24	-0.47	0.71	-0.44	-0.70	-0.66	-0.43	1.99
25	1.05	0.87	1.27	-0.81	-0.74	-0.81	-0.81
26	0.06	1.94	-0.67	-0.60	-0.83	-0.54	0.64
27	0.01	1.70	-0.78	-0.68	-0.78	-0.54	1.07
28	1.22		-0.71	-0.63	-1.03	-0.08	1.24
29	0.97	1.77	-0.69	-0.71	-0.81	-0.54	0.00
30	1.06		-0.51	-0.82	-0.96	-0.19	1.41

Based on the EROD z score calculations, only two samples (9 and 10) for lab code 10, had Z values marginally above 2 indicating a questionable result (Table 5). All other EROD Z scores were below the threshold of 2 indicating satisfactory results.

With respect to protein concentrations, the Z score calculations were also calculated after the removal of the outliers as identified with the Dixon Q test (Table 6). Lab code 4 had 14 Z score

values greater than 2 indicating questionable results. Other lab codes that had Z scores greater than 2 included Lab code 6 for sample 27, Lab code10 for samples 10 and 28, as well as Lab code 11 for samples 2 and 13. All other protein concentration Z scores were below the threshold of 2 indicating satisfactory results.

Table 6. Calculated z scores for the protein concentrations of the microsomal (1-15) and S9 (16-30) fractions for all 10 laboratories. Outliers based on the Dixon Q test were removed from the Z score calculation (blank cells).

sample	Lab code									
	1	2	3	4	5	6	8	9	10	11
1	0.07	-0.33	-0.77	-2.15	0.44	1.10	0.26	-0.43	0.49	1.33
2	-0.16	-0.43	-0.45	-1.75	0.21	0.61	0.32	-0.34	-0.20	2.20
3	0.09	0.01	-0.55	-2.02	0.64	0.26	0.27	-0.29	-0.37	1.96
4	0.05	-0.31	-0.78	-1.96	0.33	0.65	0.13	-0.48	0.53	1.84
5	0.37	-0.07	-0.47	-1.93	0.64	1.04	0.32	-0.32	-1.04	1.46
6	0.56	-0.86	-1.05		-0.52	1.11	0.39	-0.95	-0.42	1.76
7	0.23	-0.27	-0.70	-2.03	0.17	0.15	0.13	-0.51	1.44	1.37
8	0.07	-0.11	-0.71	-2.17	0.20	0.21	0.26	-0.27	1.70	0.83
9	0.21	-0.93	-1.61		0.97		0.74	-0.61	-0.04	1.27
10	-0.10	-0.31	-0.51	-1.79	-0.22	0.42	0.13	-0.43	2.13	0.69
11	0.09	-0.19	-0.80	-2.17	0.54	0.51	0.32	-0.50	0.70	1.50
12	-0.09	-0.15	-0.62	-2.21	0.16	0.93	0.39	-0.51	0.74	1.36
13	0.01	-0.80	-1.20		0.00	0.26	0.42	-1.02	0.18	2.14
14	-0.01	0.04	-0.70	-1.83	0.00	0.89	0.33	-1.14	1.19	1.24
15	0.25	-0.10	-0.69	-2.15	0.16	1.04	0.45	-0.32	-0.18	1.54
16	0.18	0.03	-1.07		-0.31	1.35	0.01	-0.74	1.72	-1.18
17	-0.12	-0.24	-1.14		0.27	1.32	-0.15	-0.74	1.80	-1.00
18	-0.03	-0.32	-1.21		0.27	1.21	0.14	-0.79	1.78	-1.04
19	0.26	0.04	-0.48	-2.18	0.20	1.15	0.10	-0.32	1.54	-0.32
20	-0.12	0.20	-0.95		0.55	1.85	0.10	-1.00	0.72	-1.34
21	0.31	-0.02	-0.55	-2.12	0.18	1.42	0.14	-0.28	1.34	-0.41
22	0.33	0.10	-0.32	-2.25	0.34	0.86	0.09	-0.21	1.58	-0.52
23	-0.01	-0.12	-0.99		0.19	1.32	-0.06	-0.60	1.68	-1.42
24	0.14	-0.36	-1.26		0.33	1.43	0.18	-0.72	1.46	-1.19
25	0.07	0.13	-0.03	-2.15	0.42	0.37	0.20	-0.27	1.88	-0.62
26	0.01	0.06	-0.45	-2.11	0.17	1.22	0.25	-0.36	1.58	-0.36
27	-0.20	-0.12	-0.56		0.65	2.21	0.03	-0.67	0.03	-1.37
28	0.09	0.06	-0.49	-1.82	0.04	0.61	0.11	-0.35	2.18	-0.44
29	0.21	-0.09	-0.46	-2.11	0.38	0.72	0.12	-0.34	1.84	-0.28
30	0.34	-0.03	-0.55	-2.19	0.44	1.11	0.27	-0.34	1.40	-0.46

Preliminary Conclusions

The intercalibration clearly showed that inter-laboratory differences exist in samples that have been prepared in exactly the same way. For the seven laboratories that reported EROD activity in pmol/ min/ mg protein a reasonable agreement between the reported values for the different samples was shown. Although higher values were consistently reported by lab code 2, all the seven laboratories were able to differentiate well between the control, low and high exposure groups. These laboratories also consistently reported higher EROD activity in the microsomal fractions than the S9 fractions.

Overall, these seven laboratories faired reasonably well in the EROD intercalibration exercise and based on the calculated Z score values, when excluding the outlier values based in the Dixon Q test, provided satisfactory result (Z score < 2).

For the three laboratories that reported values in nmol/ min/ mg protein (lab codes 4, 6 and 11), lab code 4 was not able to differentiate between the treatment groups and reported relatively low EROD activity for all samples. Lab codes 6 and 11 were able to differentiate between the exposures, particularly between the control and high exposure treatments.

With exception to Lab Code 4, which reported much lower concentrations of protein in all samples than the other labs, the protein concentrations between the labs were fairly similar and predominantly lay within the range 15 to 30 mg/ml. The protein concentrations reported by Lab code 4 were responsible for 10 outliers (Dixon Q test) as well as questionable results in 14 additional samples (Z score >2). Additionally, questionable results were identified for Lab code 6 for 1 sample, and by Lab code 10 and 11 for 2 separate samples.

Main findings of the questionnaire

Despite the reasonable agreement in EROD activity between some of the laboratories, differences were apparent. In order to shed some light on the differences in EROD activity as well as protein determination between laboratories, a set of questions were sent out to each laboratory. Of the 10 laboratories that took part in the inter-calibration 9 laboratories responded to the questionnaire. A summary of the questionnaire including the main responses for both EROD and protein are shown in table 7 and 8.

Table 7. Summarised list of questions and lab responses from 9 of the 10 participating laboratories that took part in the EROD intercalibration.

Questions on EROD	Lab responses
Which EROD method was used?	2 labs x TIMES 57, 1 lab x TIMES13, 3 labs x TIMES23, 2 other.
Homogenisation protocol: amount of tissue to buffer, time from thawing the liver to freezing S9/ microsomal fraction	Identical by UGOT
How many samples are processed or defrosted at the same time? (i.e., batch size)	1, 1, 12, 30, 5, 15, 20, 15, 10
What is the estimated time from beginning to end of thawing your sample until reading it?	30 sec, 3-5 min, 5 min, 20-25 min, 30 min, 30 min, 1 h, 2 h, 2 h.
Was EROD activity measured more than once on the same (previously defrosted) sample?	6 YES, 3 NO
Did saturation occur in the EROD kinetics analysis?	5 YES, 4 NO
Are you using a cuvette, microplate (96, 48, 24 well)?	3 cuvettes, 5 x 96 well plate, 1 lab 48 well plate and cuvette
How was EROD activity reported? nanomoles or picomoles?	3 nano, 6 pico
For how long and how frequent was the EROD activity kinetics measured?	1 min, 1 min, 8 x 40 sec intervals, 6 x 5 min, 1 min, 7 min, 1 min, 2 min, 20 sec intervals over 5 min.
Do you include quenched samples in your analysis.	1 YES, 8 NO
Which excitation-emission wavelengths do you measure for the EROD activity?	(2 x Ex535 Em585), (2 x Ex530 Em590), (2 x Ex535 Em580), (3 x Ex530 Em585)

The questionnaire revealed several interesting findings that provided insight into some of the possible reasons for the differences in EROD activity reported between the laboratories. One important finding was the difference in batch size, which ranged from 1 to 30. The problem with larger batch sizes being that the samples are thawing on ice and must wait to be analysed, potentially losing activity over time. Therefore, the same sample that is analysed first in the batch compared to being analysed last, would potentially have a higher activity. For this reason, it is recommended that batch sizes are kept to a minimum.

Connected to the question on batch size is the question of the time taken from beginning to end of thawing the sample until it being measured. Laboratory responses included from 30 seconds to 2 hours. Such large time differences would likely lead to differences in activity. A sample that is left on ice for 2 hours is likely to have much lower enzyme activity, when eventually being measured, compared to the same sample measured within 30 seconds of thawing.

Although there were other differences in response to the questions, the batch size and time taken from thawing to analysing appeared to provide the clearest explanation for the differences observed. Other factors highlighted from the questionnaire include: 1) different protocols being used; 2) the frequency and period of time the EROD activity kinetics was measured; 3) whether EROD activity was measured more than once; 4) whether a cuvette or microplate is used; and 5) the slight differences in excitation-emission wavelengths that EROD activity was measured.

It should be acknowledged that some misunderstanding and/ or interpretation of the questions may have occurred to a certain extent. However, despite this, the questionnaire provided important insights into the different methodological approaches used by the laboratories.

Table 8. Summarised list of questions and lab responses from 9 of the 10 participating laboratories that performed the protein measurements of the prepare liver samples.

Questions on Protein measurements	**Lab responses**
Do you use "ready-to-use" solutions from Bio-Rad or prepare your own solutions?	6 x Bradford with Biorad
Incubation times during analysis?	30 min then 50 min, 10 and 30 min Bradford with BioRad (15, 5, 30, 15, 0, 15 min)
What do you use to dilute your samples?	5 x MilliQ, Tris buffer, phosphate buffer
At what temperature are you diluting and storing your samples until analysis?	2 x Dilute using milliQ at RT 5 x -80C, 1 x on ice
At what temperature are your solutions when diluting your samples? Are they on ice, fridge cold, or at room temperature?	5 x RT, 3 x on ice
Which protein standard	1 x Bovine gamma globulin (BGG) 7 x Bovine Serum Albumin (BSA)
Do you weigh out the standard yourself or is it already pre-weighed (ready-to-use)?	2 x use pre-weighed 6 x weigh out themself
Are you using a Cuvettes, microplate (e.g., 96, 48, 24 well)?	1 x cuvette 7 x 96 well microplate
How many replicates?	1x duplicate, 6 x triplicate, 1 x quadruplicate
Do you measure a standard on each plate?	5 x YES, 3 x NO
Which excitation (absorption) -emission wavelengths do you measure for the protein?	2 x 750 nm, 1 x 690 nm, 3 x 595 nm, 1 x 590 nm, 1 x at 550 nm

References

ICES TIMES 13: Galgani, F.; Payne, J. F. (1991). Biological effects of contaminants: Microplate method for measurement of ethoxyresorufin-O-deethylase (EROD) in fish. ICES Techniques in Marine Environmental Science (TIMES). Report. https://doi.org/10.17895/ices.pub.5039

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