| Measurement of the polycyclic aromatic hydrocarbon metabolite 1-hydroxypyrene in fish bile<br>Inter-laboratory calibration<br><br>FINAL REPORT |  <br>BEQUALM<br>Biomarker Programme Office<br>NIVA, Gaustadalléen 21, NO-0349, Oslo, Norway |
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## 1. Introduction

The Biological Effects Quality Assurance in Monitoring (BEQUALM) programme was developed as a quality assurance (QA) system for biological effects techniques that are used in national and international monitoring programmes. BEQUALM consists of three main components of which 'Biomarkers' is one of these components that is currently led by the Norwegian Institute for Water Research (NIVA).

Biomarkers to be used for national or international monitoring programmes should be subject to appropriate internal and external Analytical Quality Control (AQC), to ensure results produced are comparable with other laboratories. This report describes an inter-calibration exercise measuring polycyclic aromatic hydrocarbon (PAH) metabolites in fish bile. PAH metabolites are widely used within European environmental laboratories providing an indication of exposure to PAH chemicals and considered a core biomarker within the ICES integrated monitoring and assessment framework (Davies and Vethaak, 2012). The PAH metabolite measured by all participating laboratories was 1-hydroxypyrene, whilst 1-hydroxyphenathrene was also measured by three laboratories and is included in this report.

## 2. Participating laboratories

Eleven laboratories registered to take part in the intercalibration exercise and received samples for analysis. However, for different reasons only 8 laboratories were able to submit their data and are presented in this report. The laboratories were identified by individual laboratory codes in order to keep the intercalibration anonymous.

## 3. Approach

### 3.1. Test material

Test material, consisting of 9 fish bile samples from coastal and offshore monitoring programmes in Norway were used in the intercalibration (Table 1). These bile samples were collected from fish that were killed with a blow to the head and the bile immediately removed from the gall bladder with a disposable syringe. The bile samples were stored at -20°C until required. In order to obtain sufficient sample volume for distribution, the bile samples were pooled from several individuals.

Table 1. Information on the source of the fish bile samples for the intercalibration and the volume of sample sent to each participating laboratory. Samples were obtained from the Norwegian coastal (MILKYS, Green et al., 2015) and offshore (WCM 2017, Pampanin et al., 2017) monitoring programmes.

| Sample No. | Station | Species | Samples used in pooled samples | Location in Norway | Vol. (µl) |
|---|---|---|---|---|---|
| 1 | MILKYS 2015 - 23B | Cod | 15 fish (23B 1-15) | Bømlo_ west coastal | 200 |
| 2 | MILKYS 2015 - 30B | Cod | 30B 1-15 | inner Oslofjord | 200 |
| 3 | MILKYS 2015 - 53B | Cod | 53B 1-15 | Sørfjord - west coast | 150 |
| 4 | MILKYS 2015 - 15B | Cod | 15B 1-15 | Farsund, SW coastal | 200 |
| 5 | WCM2017-Tampen | Ling/Sei | 5 | Tampen region, North Sea | 180 |
| 6 | WCM 2017 - "Ling" | Ling/Sei | #47, #49, #52, #58, #63, #87, #97, #98 | Statfjord A platform | 200 |
| 7 | MILKYS 2015 - 23B | Cod | 23B 1-15 | Bømlo_ west coastal | 200 |
| 8 | WCM 2017 - MixPool | Pollock, cod, tusk, hake, whiting, saithe | #29, #89, #90, #23, #35, #59, #60, #66, #82 | Statfjord A platform | 200 |
| 9 | Pool of MILKYS 2015 | Cod | 23B 1-15; 30B 1-15; 53B 1-15; 15B 1-15 | mixed coastal | 200 |

### 3.2. Sample preparation and distribution

All samples were homogenised by intensive shaking with a vortexer and immediately aliquoted 150 – 200 µl into separate microcentrifuge tubes. Each laboratory was sent one aliquot of each of the 9 samples. The samples were transported on dry ice by courier. All samples arrived at their destinations frozen, with adequate amounts of dry ice remaining.

### 3.3. Analytical methods

Each participating laboratory used their own method for the determination of PAH metabolites in the fish bile samples. Three methods were used including high performance liquid chromatography (HPLC), synchronous fluorescence spectrometry (SFS) and fixed wavelength fluorescence (FF). The main details of each protocol, provided by the participating laboratories, are shown in Table 2.

### 3.4. Data assessment

For a statistical comparison between the participating laboratories for the PAH metabolite concentrations of the nine samples, individual z scores were calculated. The z scores were calculated using the formula:

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

An assessment criterion for each z score was based on the ISO/IEC 17043:2010 guidelines:

| | |
|---|---|
| z score < 2 | satisfactory |
| 2 < z score < 3 | questionable |
| z score > 3 | unsatisfactory |
| z score > 6 | extreme |

Table 2. Overview of the methods used by each laboratory for the analysis of 1-hydroxypyrene in fish bile samples.

| Lab code | Method | Sample preparation | Chromatographic separation | Detection | Calibration |
|---|---|---|---|---|---|
| 1 | HPLC-F | enzymatic hydrolysis, ethanol addition, centrifugation | RP-18 column: Multosphere 100-3 C18, 3 x 125 mm, gradient of 0.1% TFA and ACN (50-100%) | Ex/Em 256/380nm, 346/384nm | external standard |
| 2 | HPLC-F | enzymatic hydrolysis, dilution in methanol | C-18, mobile phase: gradient with ACN and 0,05 M ammonium acetate buffer | HPLC-F | internal standard |
| 3 | HPLC-F | enzymatic hydrolysis | HPLC | Fluorescence | External calibration with TPA as internal standard |
| 4 | HPLC-MS/MS | Diluted in acetate buffer pH5, enzyme hydrolysis (ß-glucuronidase, 2-mercaptoethanol, 37°C, 16h), extraction using SPE 500mg-C18 (methanol) and purification using SPE 500mg-NH2 (80/20 v/v dichloromethane/methanol) | Chromatographic column: Acquity UPLC BEH C18 (1.7µm x 2.1mm x 50mm, Waters). | | Internal standards: 1 hydroxypyrene d9 |
| 5 | SFS | Diluted 1:1000 in 48% ethanol | NA | Fluorescence | Internal standard |
| 6 | SFS | Diluted 50:50 nanopure water: ethanol | NA | Fluorescence | Internal standard |
| 7 | FF | methanol | NA | Fluorescence 341/383 | |
| 8 | FF | ethanol 50% | NA | Fluorescence 341/383 | Internal standard |

## 4. Results and discussion

Data were submitted from all 8 laboratories for 1-hydroxypyrene, whilst 3 laboratories submitted data for 1-hydroxyphenanthrene.

### 4.1. 1-hydroxypyrene

Four laboratories submitted data for 1-hydroxypyrene using HPLC, whilst two laboratories used SFS and two further laboratories used FF (see Table 3). To adjust for the difference in fluorescence intensity between conjugated and nonconjugated pyrene metabolites the SFS data were divided by 2.2 (Ariese et al. 1993, Tairova et al. 2012). This would enable the SFS and HPLC data to be compared. No conversion factor was available for the pyrene data determined by FF, and the two data sets submitted using FF were not included in the wider comparison.

For the 1-hydroxypyrene concentrations measured by FF, there was good agreement between the two laboratories, although these values were typically two orders of magnitude lower than the values reported by laboratories using HPLC and SFS methods.

Table 3. Data for 1-hydroxypyrene submitted by each laboratory for the nine samples including the method used for analysis (ng/ml).

| Sample | HPLC | HPLC | HPLC | HPLC | SFS* | SFS* | FF | FF |
|---|---|---|---|---|---|---|---|---|
| | Lab 1 | Lab 2 | Lab 3 | Lab 4 | Lab 5 | Lab 6 | Lab 7 | Lab 8 |
| **1** | 26.00 | 21.34 | 24.30 | 14.90 | 49.87 | 62.99 | 0.56 | 0.21 |
| **2** | 268.20 | 247.83 | 249.00 | 213.40 | 545.57 | 463.04 | 5.00 | 3.13 |
| **3** | 83.80 | 97.75 | 99.40 | 76.00 | 240.22 | 259.19 | 2.80 | 1.81 |
| **4** | 140.90 | 266.65 | 289.00 | 203.80 | 714.61 | 377.01 | 3.60 | 2.86 |
| **5** | 1.90 | 2.00 | 3.98 | 1.90 | 12.65 | 29.06 | 0.39 | 0.18 |
| **6** | 2.30 | 3.08 | 4.18 | 1.90 | 10.91 | 28.15 | 0.26 | 0.07 |
| **7** | 27.70 | 21.18 | 23.20 | 18.50 | 41.13 | 61.15 | 0.71 | 0.15 |
| **8** | 12.00 | 7.75 | 9.67 | 6.90 | 63.73 | 81.26 | 1.20 | 0.18 |
| **9** | 211.30 | 171.18 | 192.00 | 148.10 | 752.30 | 311.97 | 3.20 | 2.17 |

* SFS divided by 2.2 as described by Ariese et al., (1993).

The calculated z scores for 1-hydroxypyrene measured by HPLC and SFS for the nine samples are shown in Table **4** and in Figure 1. All z score values were below the benchmark z value of 2 and were considered satisfactory. However, despite applying the conversion factor of 2.2 to the SFS data, the measured values were consistently higher than that measured by the HPLC method. For the SFS method, z score values were typically above 1 and approaching 2 in samples 4 and 9 for laboratory 5 and samples 5 and 6 for laboratory 6.

The data submitted by the four laboratories that used the HPLC method were very similar to each other and all samples had calculated z values within 1 unit.

Table 4. Comparison of 1-hydroxyprene values and calculated z scores for each sample. Data for HPLC and SFS only.

| **Sample** | Method | **HPLC-F** | **HPLC-F** | **HPLC-F** | **HPLC-MS** | **SFS** | **SFS** |
|---|---|---|---|---|---|---|---|
| | Lab code | 1 | 2 | 3 | 4 | 5 | 6 |
| **1** | ng/ml | 26.00 | 21.34 | 24.30 | 14.90 | 49.87 | 62.99 |
| | z score | -0.38 | -0.63 | -0.47 | -0.97 | 0.88 | 1.58 |
| **2** | ng/ml | 268.20 | 247.83 | 249.00 | 213.40 | 545.57 | 463.04 |
| | z score | -0.46 | -0.60 | -0.60 | -0.85 | 1.56 | 0.96 |
| **3** | ng/ml | 83.80 | 97.75 | 99.40 | 76.00 | 240.22 | 259.19 |
| | z score | -0.71 | -0.54 | -0.52 | -0.80 | 1.17 | 1.39 |
| **4** | ng/ml | 140.90 | 266.65 | 289.00 | 203.80 | 714.61 | 377.01 |
| | z score | -0.94 | -0.32 | -0.21 | -0.63 | 1.88 | 0.22 |
| **5** | ng/ml | 1.90 | 2.00 | 3.98 | 1.90 | 12.65 | 29.06 |
| | z score | -0.62 | -0.61 | -0.42 | -0.62 | 0.37 | 1.89 |
| **6** | ng/ml | 2.30 | 3.08 | 4.18 | 1.90 | 10.91 | 28.15 |
| | z score | -0.60 | -0.52 | -0.42 | -0.64 | 0.24 | 1.93 |
| **7** | ng/ml | 27.70 | 21.18 | 23.20 | 18.50 | 41.13 | 61.15 |
| | z score | -0.27 | -0.67 | -0.55 | -0.84 | 0.55 | 1.78 |
| **8** | ng/ml | 12.00 | 7.75 | 9.67 | 6.90 | 63.73 | 81.26 |
| | z score | -0.55 | -0.68 | -0.62 | -0.70 | 1.01 | 1.53 |
| **9** | ng/ml | 211.30 | 171.18 | 192.00 | 148.10 | 752.30 | 311.97 |
| | z score | -0.38 | -0.55 | -0.46 | -0.65 | 1.98 | 0.06 |

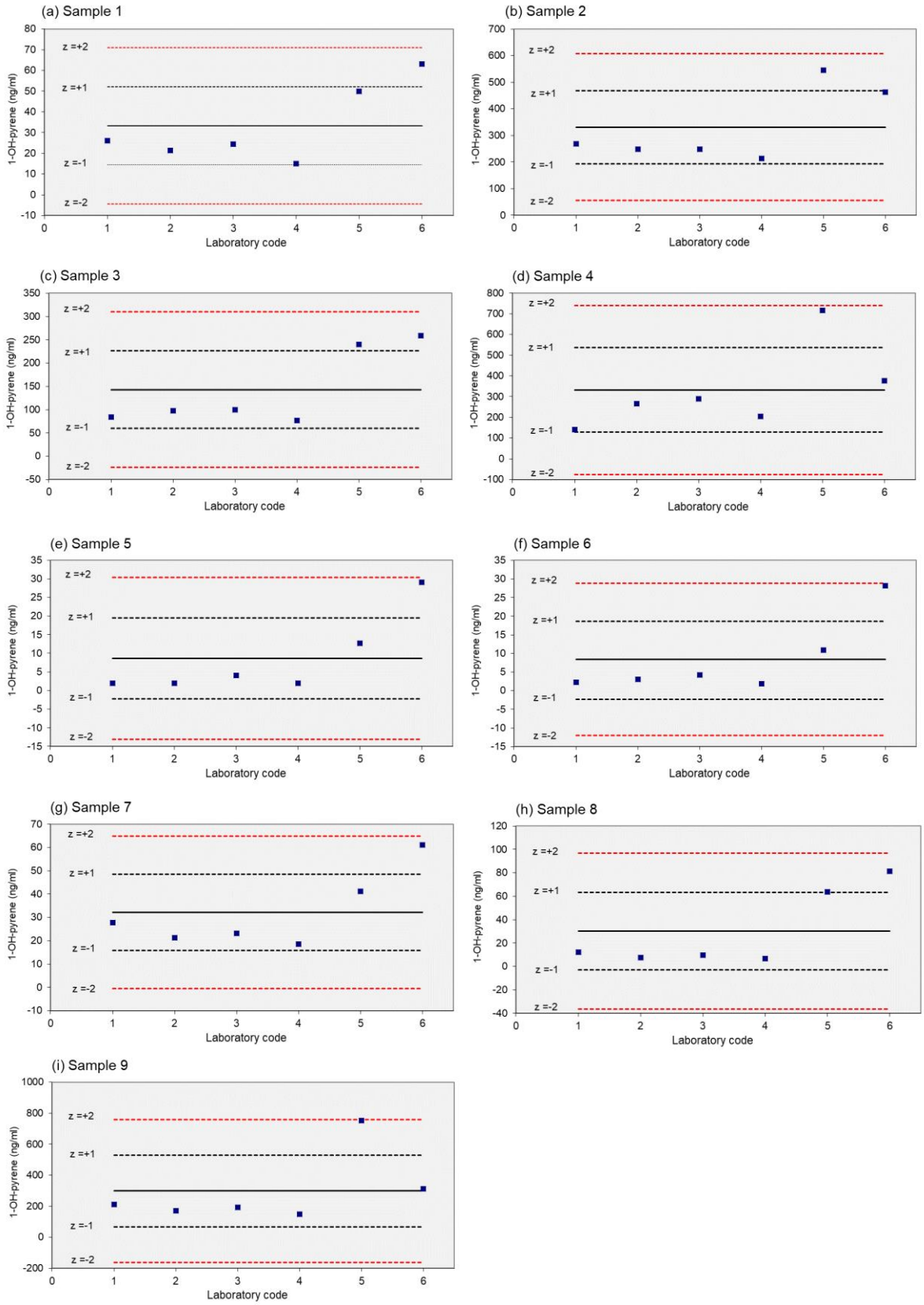


Figure 1. Concentrations of 1-hydroxypyrene in the nine bile samples measured using HPLC (labs 1-4) and SFS (labs 5-6). Note SFS values were divided by a conversion factor of 2.2 (Ariese et al., 1993).

### 4.2. 1-hydroxyphenanthrene

Three laboratories submitted data on 1-hydroxyphenanthrene for all nine bile samples by HPLC (Table 5, Figure 2). Although differences were observed between the laboratories for the nine samples, all z scores were found within or slightly outside ± 1 unit indicating satisfactory results.

Table 5. Comparison of 1-hydroxyphenanthrene values and calculated z scores for each sample from the three laboratories.

| sample | Method | HPLC-F | HPLC-F | HPLC-MS |
|---|---|---|---|---|
| | Lab code | 1 | 2 | 4 |
| **1** | ng/ml | 6.00 | 4.60 | 1.70 |
| | z score | 0.87 | 0.23 | -1.09 |
| **2** | ng/ml | 7.20 | 0.50 | 0.50 |
| | z score | 1.15 | -0.58 | -0.58 |
| **3** | ng/ml | 5.30 | 5.98 | 4.70 |
| | z score | -0.04 | 1.02 | -0.98 |
| **4** | ng/ml | 8.30 | 11.55 | 7.80 |
| | z score | -0.45 | 1.15 | -0.70 |
| **5** | ng/ml | 0.10 | 1.00 | 0.40 |
| | z score | -0.87 | 1.09 | -0.22 |
| **6** | ng/ml | 4.50 | 0.72 | 0.10 |
| | z score | 1.15 | -0.44 | -0.70 |
| **7** | ng/ml | 7.30 | 3.81 | 1.50 |
| | z score | 1.06 | -0.13 | -0.93 |
| **8** | ng/ml | 7.10 | 2.30 | 0.60 |
| | z score | 1.12 | -0.31 | -0.81 |
| **9** | ng/ml | 6.90 | 5.08 | 2.70 |
| | z score | 0.95 | 0.09 | -1.04 |

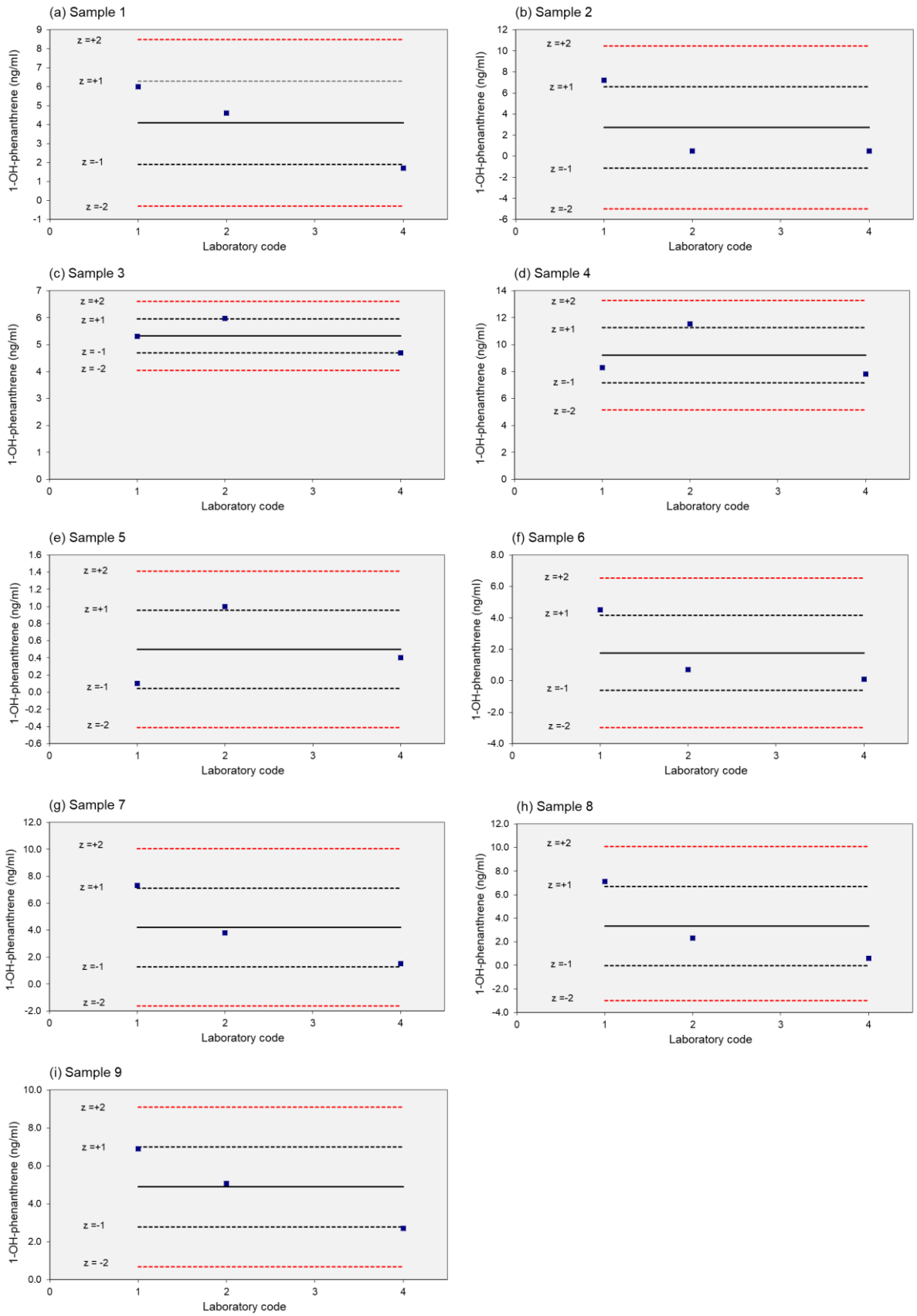


Figure 2. Concentrations of 1-hydroxyphenanthrene in the nine bile samples measured using HPLC by three laboratories.

## 5. Conclusions

- Data for 1-hydroxypyrene were submitted by 8 laboratories, where 4 used the method of HPLC, 2 used SFS and 2 used FF. A conversion factor of 2.2 was applied to the SFS data in order to enable comparison between the HPLC and SFS values.
- FF was not comparable to the other methods and was not used in the wider intercalibration. FF values were two orders of magnitude lower than HPLC and SFS, although between-laboratory variability for the FF method was low.
- Interlaboratory comparison (6 laboratories) of 1-hydroxypyrene for the nine bile samples using both the HPLC and SFS methods was considered satisfactory with z scores within ± 2 units.
- Interlaboratory comparison (3 laboratories) of 1-hydroxyphenanthrene for the nine bile samples using HPLC was also considered satisfactory with z scores within ± 2 units.

## 6. References

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