Application of the EROD-H4IIE bioassay for the determination of dioxins in pork in comparison to high resolution gas chromatography coupled to high resolution mass spectrometry*

Aplicación del bioensayo EROD-H4IIE para la determinación de dioxinas en carnes de cerdo en comparación con la cromatografía de gases de alta resolución acoplada a espectrometría de masas de alta resolución

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RESUMEN
El bioensayo EROD-H4IIE se utiliza en la detección de hidrocarburos halogenudos planares, presentando ventajas sobre la química analítica, como velocidad, simplicidad, precisión, alta sensibilidad y bajo costo, siendo un valioso bioensayo para estudios de biomonitorio. Se ha aplicado exitosamente como método de cribado para la detección de dioxinas y furanos (PCDD/Fs) en carne de pollos broiler. En el presente trabajo realizamos un estudio para detectar PCDD/Fs en carne de cerdo, aplicando el bioensayo y la cromatografía de gases acoplada a espectrometría de masas de alta resolución (HRGC/HRMS). Se tomaron 59 muestras compuestas de lomo vetado de cerdo en la planta faenadora de 6 planteles de Chile, durante el beneficio de animales entre 2004 y 2007 y una muestra fue tomada en 2011. Los concentraciones promedio de PCDD/Fs obtenidas mediante HRGC/HRMS oscilaron entre 0,22 y 0,34 pg WHO-TEQ/g de grasa. El contenido máximo fue 0,71 pg/g de grasa. El mayor valor promedio de los congéneres fue 0,1 pg/g de grasa de 2,3,4,7,8-PeCDF (entre 0,03 y 0,28 pg/g de grasa). Por tanto, las muestras no excedieron los límites máximos permitidos por la legislación nacional e internacional. Comparando ambos métodos, de las 59 muestras el bioensayo sobrestimó 36 resultados, subestimó 19 y 4 fueron similares. 15 muestras excedieron 1 pg TCDD-EQ/g de tejido y 5 superaron los 2 pg TCDD-EQ/g de tejido. No se estableció una equivalencia entre EROD-H4IIE y HRGC/HRMS para la detección de PCDD/Fs en carne de cerdo, no existiendo asociación significativa entre las variables (r = -0,142, P ≥ 0,2840).

Palabras clave: dioxinas, EROD/H4IIE, HRGC/HRMS, carne de cerdo.

SUMMARY
The EROD-H4IIE cell bioassay is a screening method for the detection of planar halogenated hydrocarbons (PHH), it has several advantages over analytical chemistry like speed, simplicity, accuracy, high sensitivity and low cost which makes it a valuable bioassay for biomonitoring studies. It has been successfully used as screening method for the detection of dioxins and furans (PCDD/Fs) in meat from broiler chicken. The aim of this study was to detect PCDD/Fs in pork, by using both the bioassay and the high resolution gas chromatography/high resolution mass spectrometry (HRGC/HRMS). 59 composite samples of pork chuck loin were taken at a slaughtering plant of 6 Chilean production facilities between 2004 and 2007, with one sample being taken in 2011. Mean concentrations of PCDD/Fs obtained by HRGC/HRMS ranged between 0.22 and 0.34 pg WHO-TEQ/g of fat. The maximum was 0.71 pg WHO-TEQ/g fat. The average value of the congeners was 0.1 pg WHO-TEQ-fat-2,3,4,7,8-PeCDF (between 0.03 and 0.28 pg/g fat). Therefore, the samples did not exceed the maximum allowed by national and international legislation. When comparing the results obtained with the two methods, 36 out of 59 sample results were overestimated by the bioassay, while 19 were underestimated and 4 were similar. 15 samples exceeded 1 pg TCDD-EQ/g tissue and only 5 exceeded 2 pg TCDD-EQ/g tissue. It was not possible to establish an equivalence between EROD-H4IIE and HRGC/HRMS for the detection of PCDD/Fs in pork since there was not a significant association between variables (r = -0.142, P ≥ 0.2840).

Key words: dioxins, EROD/H4IIE, HRGC/HRMS, pork.

INTRODUCTION
Dioxins (polychlorinated dibenzo-p-dioxins, PCDDs and dibenzofurans, PCDFs) are unwanted contaminants almost principally produced by industrial processes, including incineration (Olie 1980, USEPA 2001), pyrolysis processes, chlorine bleaching of paper and pulp, and the manufacture of some pesticides, herbicides, and fungicides (Gilpin et al 2003). Dioxins did not exist prior to industrialization except in very small amounts due to natural combustion and geological processes (Czuczwa et al 1984, Ferrario and Byrne 2000). From a total of 7,270 samples collected in the period 1999-2008 from 19 Member States of European Union, PCDD/Fs congeners

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The release of PCDD/Fs and PCBs to the circulating environment has changed so that greater proportions of dioxin congeners of lower toxicity are emitted. This pattern of decline has resulted in significant reductions in average human exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the dioxin congener of greatest toxicity and concern, and current exposures to dioxin are typically composed of smaller amounts of lower toxicity congeners (Hays and Aylward 2001, Petreas et al 2001, Hites 2011). However, the presence of dioxins has been reported in pork for human consumption (Bernard et al 2002, Guruge et al 2005, Hoffman et al 2006, Hoogenboom et al 2007, Covaci et al 2008, Kim et al 2011, PoultryMed1), raising concerns due to accumulation of these xenobiotics in the food chain, particularly in animal fat. In this sense, the European Union has adopted a series of regulatory measures to reduce the presence of PCDD/Fs, in addition to monitoring food of animal origin, including pork from the Community member states, by using high resolution gas chromatography/high resolution mass spectrometry (HRGC/HRMS). This analytical technique requires investment in infrastructure and equipment at a considerable cost as well as highly trained technicians to detect individual congeners of PCDD/Fs and DL PCBs (Valdivinos 2009, Babin et al 2010). Therefore, a number of screening assays has been developed, such as the 7-ethoxyresorufin-O-deethylase (EROD)-bioassay, the aryl hydrocarbon hydroxylase (AHH) bioassay, the enzyme immunoassay (ELA), the chemical-activated luciferase gene expression (CALUX), the gel retardation of AhR DNA binding (GRAB) assay, the recAhr DELFIA assay kit, the Ah receptor (AhR) (or filtration) assay with radiolabeled dioxins, the Ah-immunoassay (AhIA) (Behnisch et al 2001), the chemical-activated fluorescent expression (CAFLUX) (Zhao et al 2010) and DR-EcoScreen® bioassay (Anezaki et al 2009, Kojima et al 2010). A viable alternative is to use in vitro bioassay cell line H4IIE rat hepatoma as screening method in pork production, considering that Schoffer et al (2011) determined the equivalence between the results of the bioassay of meat from broiler chickens with HRGC/HRMS, showing that the first one provides very accurate estimates ($R^2=0.885$), and is therefore considered a useful technique for biomonitoring, as a screening method, in broiler chicken production and can be extended to other animal production systems. Therefore, the objective of this study is to determine the presence of dioxins and furans in pork produced in Chile by HRGC/HRMS in pg WHO-TEQ/g fat and H4IIE-EROD bioassay in pg TCDD-EQ2/g of sampling tissue, and to determine the equivalence of the results generated by the bioassay as a screening method and HRGC/HRMS as identity confirmatory method.

MATERIAL AND METHODS

SAMPLES

59 composite samples of pork were used, each sample of 1 kg was obtained from chuck loins of 10 animals from the same origin, obtained from the slaughtering plant of 6 production facilities in Chile, coded as P1, P2, P3, P4, P5 and P6, for the slaughter of animals (180 days of age) between 2004 and 2007, with one sample being taken in 2011. Samples were ground-up using an industrial meat grinder in the slaughtering plant (BESTE® TK-12), obtaining two samples of 250 g for the EROD-H4IIE bioassay and 2 samples of 250 g for the HRGC/HRMS analysis (sample and countersample). They were placed in aluminum containers, sealed and frozen at -70° C in the laboratory to pending analysis. The samples that were taken until 2007 came from export meat and the sample taken in 2011 came from meat for domestic consumption.

EXTRACTION AND PURIFICATION FOR APPLICATION OF H4IIE CELL BIOASSAY

The extraction process was performed according to the method described by Nicks and Tillitt (2003). A 20 g sample of meat was weighed and 60 g of sodium sulfate were added ($Na_2SO_4$) to dry the sample. Then each dried sample was homogenized in a blender (Osterizer® 4172). The fat extraction was performed in an automated Soxhlet (VELP® Scientifica SER 148) during 2 hours and 30 minutes, with a 1:1 mixture of dichloromethane-hexane. Determination of the lipid content of the extract was performed by gravimetric calculations. To clean the extract, the sample was first passed through a glass column filled with $Na_2SO_4$, potassium silicate (KS), silica 60:40 (Grade 62 silica gel, 60-200 mesh, 150 Å, SIGMA-ALDRICH® mixed with $H_2SO_4$) with dichloromethane. Then the extract was again exposed to a second cleaning column.

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2 TCDD-EQs is used in this document to describe the toxic equivalents (TEQs)-derived from EROD-H4IIE bioassay.
containing Na₂SO₄, silica gel 60 (Grade 60 silica gel, 70-230 mesh, 0.063-0.200 mm; Merck®), KS and silica 60:40 with a mixture of dichloromethane: hexane/3:97. The obtained sample was reduced using a rotary evaporator (Heidolph® Laborota 4000) to a volume of 1 mL. The solvent was exchanged using gaseous nitrogen to obtain a final extract of 150 µL dissolved in isoctane. All the used solvents were HPLC grade.

**EROD-H4IIE CELL BIOASSAY**

EROD-H4IIE cell bioassay was applied for the detection of PCDDs, and DL-PCBs, according to the protocol described by Tillitt *et al* (1991) and modified by Nicks and Tillitt (2003). The hepatoma cell line *Rattus norvegicus* was used (ATCC®, code CRL-1548™), it was grown in an Eagle’s basal medium modified by Dulbecco (D-MEM) enriched with 15% fetal bovine serum (FBS). Cells were maintained under standard conditions (37° C, 5% CO₂) and allowed to grow between 4 to 5 days, then they were seeded in microtiter plates Nunc® flat-bottom 96 well plate, in a volume of 300 µL/well with a density of 1.2 x 10⁴ cells/well. After 24 hours of growth the plate was dosed with the standard of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in a set of 7 serial dilutions in a 1:2 ratio (50 pg/well to 0.069 pg/well) with three replicates of each (dilution 1:2). This standard was used to generate a dose-response curve according to which all samples were compared. The extracts of the samples and the quality control samples with 3 replicates each were dosed in a set of 7 in a 1:3 ratio. Similarly, dosed along with these samples were a positive control (PC) corresponding to tissue of *Cyprinus carpio* Linnaeus and a negative control or blank matrix (BM) of *Lepomis macrochirus* Rafinesque, reference materials of the Columbia Environmental Research Center, Missouri, USA. Furthermore, a procedure blank (PB) corresponding to a solution of dichloromethane-hexane 1:1 (HPLC grade) was used. After the dosage procedure, the cells were left under standard conditions for 72 hours before the reading, which was performed by removing the medium using a Thermo Weelwash® 4 Mk 2 plate washer, with ultrapure water, leaving approximately 60 µL of medium/well. After washing, the cells were incubated for 5 minutes, causing osmotic cytolyis, resulting in the exposure of cytochrome P-4501A1 (CYP1A1). Afterwards, the plate was removed from the incubator and 20 µL of buffer at 37° C with 80 µL of dicumarol were added. Subsequently, 20 µL of 5 mM of ethoxyresorufin and 20 µL of 5 µM NADPH were added. Finally, plates were placed in a fluorescence reader (BioTek® FLx800 ™) that performs readings for 20 minutes with an excitation filter of 530 nm and an emission filter of 580 nm. This method allows reading of EROD activity, as well as the protein reading in the same well (Kennedy and Jones 1994). In the final stage of the assay, dose-response curves were used to determine the relative potency of the extract (RPFs), comparing slopes values of the sample extracts with values of the standard TCDD slope to obtain toxic equivalents (TCDD-EQs) expressed in pg/g of sample (Mason *et al* 1985, Tillitt *et al* 1993, Whyte *et al* 2004). The recommendations of Whyte *et al* (2004) were considered for quality control of the bioassay, such as the use of a composite TCDD dose-response curve from the average of four independent determinations, the extraction in triplicate in at least the 5% of the analysed samples, the previously mentioned use of quality control samples (PC, MB and PB), and checking concentrations of resorufin, ethoxyresorufin, and NADPH reagents on each assay date using spectrophotometer.

**HIGH RESOLUTION GAS CHROMATOGRAPHY COUPLED TO HIGH RESOLUTION MASS SPECTROMETRY**

The analysis of 58 samples by HRGC/HRMS was performed by the Research and Productivity Council (RPC), New Brunswick, Canada. Two methods were used for this purpose: U.S. EPA Method 1613B, “Tetra-through Octa-Chlorinated Dioxins and Furans by Isotope Dilution HRGC/HRMS” and U.S. EPA Method 8290A, “Polychlorinated Dibenzo-dioxins (PCDDs) and Polychlorinated Dibenzo-furans (PCDFs) by High-Resolution Gas Chromatography/High-Resolution Mass Spectrometry (HRGC/HRMS)”, each with slight modifications (RPC Standard Operation Procedures DX09 and DX08). Dioxin-like PCBs (DL-PCBs) were not analysed in this study.

The sample (20 to 60 g) was enriched with a solution containing specified amounts of each of the 15 internal standards of isotopically labeled PCDD/Fs (Wellington Laboratories Inc., Guelph, Ontario, Canada). Soxhlet extraction was performed for 16 h with dichloromethane/hexane 1:1 (GC grade), then extracts were concentrated in a rotary evaporator (Buchi® RE 121 or Yamato® RE47). The fat content was determined by gravimetric analysis, the extracts were dissolved in hexane (GC grade). Afterwards a digestion was performed using silica 70:30 (silica gel 60. 100-200 particle size; Caledon® mixed with H₂SO₄). Then the extracts were cleaned up using a silica column of acidic/basic/silver nitrate (AgNO₃) and then using a second silica column with activated charcoal. When there was interference with chlorinated diphenyl ethers, sample extracts were cleaned up on a third basic alumina column. The final extract was enriched with a solution containing specified amounts of two isotopically labeled PCDD recovery standards (Wellington Laboratories Inc., Guelph, Ontario, Canada). A final volume of 20 micro µL was obtained. The HRGC/HRMS analysis was performed with a Hewlett Packard® HP 5890 series II gas chromatograph, coupled with VG Autospec high resolution®, mass spectrometer, resolution >10⁴, with selected multi-group ion monitoring; 1 µL injection volume; gas chromatography column Rtx-Dioxin2 60 m x 0.25 mm x 0.25 µm (Restek). Quantifica-
tion was performed using internal standards and the results were corrected for recoveries of internal standards.

The sample taken in 2011 was analysed in the Laboratory of Veterinary Pharmacology, Faculty of Veterinary Science of the Universidad de Chile (FARMAVET), applying similar analytical methods based on the USEPA protocols mentioned above. RPC’s Analytical Services laboratories hold accreditation with the Standards Council of Canada (SCC) and conform to the requirements of ISO/IEC 17025. FARMAVET is accredited ISO 17025 by Instituto Nacional de Normalización (INN), which is responsible of the accreditation in Chile. Both laboratories used the toxicity equivalence factors (TEF) established by the World Health Organization (WHO), to calculate the total concentration of dioxin-like compounds expressed in toxic equivalents or WHO-TEQ (van den Berg et al. 1998).

STATISTICAL ANALYSIS

The statistical software Minitab® version 15.0 for Windows (Minitab Inc., State College, PA, USA) was used. After obtaining the results expressed in pg WHO-TEQ/g fat for HRGC/HRMS and TCDD-EQs pg/g of tissue for EROD-H4IIE, a descriptive analysis of the data was performed for a subsequent correlation and regression analysis.

RESULTS

RESULTS OF HRGC/HRMS ANALYSIS

Table 1 shows the descriptive statistics of the results of sample analysis by HRGC/HRMS, which had low concentrations of PCDD/Fs and did not exceed the maximum allowed by Chilean legislation and the Republic of Korea of 2 pg WHO-TEQ/g fat (Minsalud 2008, Kim et al. 2011) and the maximum allowed by the European of 1 pg WHO-TEQ/g fat (EC 2006). The average of these concentrations ranged from 0.22 pg/g fat (P5) and 0.34 pg/g fat (P2). The maximum content of dioxins amounting to 0.71 pg/g fat was observed in a sample of P2, however, this value did not exceed the maximum levels.

Regarding the congeners, when they were not detected by chromatographic analysis, the corresponding calculation in WHO-TEQ was performed using the upper bound concept, which considers the limit quantification for the contribution of each non quantified congener (EC 2012). Table 2 presents the mean concentrations of congeners in pg WHO-TEQ/g fat, with the highest average value 0.1 pg/g of 2,3,4,7,8-PeCDF (range between 0.03 and 0.28 pg/g fat) in P2 samples. The second highest average concentration corresponded to 1,2,3,7,8-PeCDD (one of the most toxic congener) with 0.091 pg/g fat (range between 0.05 and 0.14 pg/g fat). OCDD and OCDF (the less toxic congeners) presented the highest average concentrations in P1 with 0.00014 and 0.00226 pg/g fat respectively. Finally, no sample exceeded the action level concentration for pork (0.75 pg/g fat) established as a European Commission Recommendation, as part of an overall strategy to reduce the presence of PCDD/Fs and PCBs in the environment, feed and foodstuffs (EC 2011).

Table 1. Descriptive statistics: HRGC/HRMS dataset in pg WHO-TEQ/g of fat.

<table>
<thead>
<tr>
<th>Production Facility</th>
<th>n</th>
<th>Mean</th>
<th>SD</th>
<th>Min</th>
<th>P25</th>
<th>P50</th>
<th>P75</th>
<th>Max</th>
<th>CV(%)</th>
<th>CI 95%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8</td>
<td>0.24</td>
<td>0.06</td>
<td>0.17</td>
<td>0.19</td>
<td>0.23</td>
<td>0.28</td>
<td>0.33</td>
<td>21.73</td>
<td>0.18 - 0.28</td>
</tr>
<tr>
<td>2</td>
<td>11</td>
<td>0.34</td>
<td>0.18</td>
<td>0.18</td>
<td>0.20</td>
<td>0.27</td>
<td>0.55</td>
<td>0.71</td>
<td>52.94</td>
<td>0.21 - 0.46</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>0.32</td>
<td>0.11</td>
<td>0.19</td>
<td>0.26</td>
<td>0.27</td>
<td>0.40</td>
<td>0.52</td>
<td>34.37</td>
<td>0.23 - 0.41</td>
</tr>
<tr>
<td>4</td>
<td>7</td>
<td>0.27</td>
<td>0.07</td>
<td>0.21</td>
<td>0.21</td>
<td>0.23</td>
<td>0.37</td>
<td>0.38</td>
<td>25.92</td>
<td>0.20 - 0.34</td>
</tr>
<tr>
<td>5</td>
<td>7</td>
<td>0.22</td>
<td>0.09</td>
<td>0.12</td>
<td>0.15</td>
<td>0.21</td>
<td>0.34</td>
<td>0.35</td>
<td>40.90</td>
<td>0.14 - 0.31</td>
</tr>
<tr>
<td>6</td>
<td>18</td>
<td>0.23</td>
<td>0.10</td>
<td>0.07</td>
<td>0.15</td>
<td>0.21</td>
<td>0.29</td>
<td>0.48</td>
<td>43.47</td>
<td>0.17 - 0.28</td>
</tr>
</tbody>
</table>

COMPARISON OF HRGC/HRMS AND H4IIE-EROD RESULTS

Table 3 shows an overestimation of the average results obtained by H4IIE/EROD bioassay compared to those obtained by HRGC/HRMS, however, when comparing the results of the 59 bioassay samples, 36 results were overestimated, 19 were underestimated and only four were similar. Figure 1 shows that both overestimations as underestimations of results were reported in samples from all production facilities. The highest bioassay overestimates were obtained with 3.25 pg TCDD-EQ/g of tissue compared to 0.21 pg WHO-TEQ/g of fat and 3.13 pg TCDD-EQ/g tissue compared to 0.20 pg WHO-TEQ/g of fat. On other hand, 15 samples exceeded 1 pg TCDD-EQ/g of tissue and only 5 exceeded 2 pg TCDD-EQ/g tissue. The highest bioassay underestimation was 0.08 pg TCDD-EQ/g tissue, compared to 0.52 pg WHO-TEQ/g fat measured with HRGC/HRMS.

Finally, the correlation analysis of the results obtained by both methods, indicates no significant association between variables (r = -0.142, P ≥ 0.2840), therefore it was not possible to establish equivalence between HRGC/HRMS and EROD-H4IIE for the detection of dioxins in pork (figure 2).
Table 2. Average of dioxin and furan congeners in pg WHO-TEQ/g of fat in samples of pork from each production facility (P). The minimum and maximum values are shown between parentheses.

Promedios de congéneres de dioxinas y furanos en pg WHO-TEQ/g de grasa en las muestras de carne de cerdo de cada plantel (P). Los valores mínimos y máximos se muestran entre paréntesis.

<table>
<thead>
<tr>
<th>PCDD/F</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
<th>P4</th>
<th>P5</th>
<th>P6</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3,7,8-TCDD</td>
<td>0.044</td>
<td>0.065</td>
<td>0.067</td>
<td>0.042</td>
<td>0.049</td>
<td>0.051</td>
</tr>
<tr>
<td>(0.010-0.070)</td>
<td>(0.030-0.090)</td>
<td>(0.040-0.090)</td>
<td>(0.05-0.09)</td>
<td>(0.01-0.11)</td>
<td>(0.01-0.13)</td>
<td></td>
</tr>
<tr>
<td>1,2,3,7,8-PeCDD</td>
<td>0.048</td>
<td>0.08</td>
<td>0.091</td>
<td>0.071</td>
<td>0.081</td>
<td>0.073</td>
</tr>
<tr>
<td>(0.001-0.080)</td>
<td>(0.030-0.170)</td>
<td>(0.050-0.140)</td>
<td>(0.05-0.11)</td>
<td>(0.03-0.23)</td>
<td>(0.01-0.18)</td>
<td></td>
</tr>
<tr>
<td>1,2,3,4,7,8-HxCDD</td>
<td>0.005</td>
<td>0.008</td>
<td>0.007</td>
<td>0.006</td>
<td>0.031</td>
<td>0.005</td>
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<tr>
<td>(0.001-0.007)</td>
<td>(0.002-0.017)</td>
<td>(0.004-0.014)</td>
<td>(0.004-0.015)</td>
<td>(0.004-0.19)</td>
<td>(0.001-0.009)</td>
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</tr>
<tr>
<td>1,2,3,6,7,8-HxCDD</td>
<td>0.005</td>
<td>0.007</td>
<td>0.008</td>
<td>0.006</td>
<td>0.006</td>
<td>0.005</td>
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<tr>
<td>(0.001-0.007)</td>
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<td>(0.004-0.016)</td>
<td>(0.004-0.015)</td>
<td>(0.003-0.009)</td>
<td>(0.001-0.013)</td>
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</tr>
<tr>
<td>1,2,3,7,8,9-HxCDD</td>
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<td>0.005</td>
<td>0.008</td>
<td>0.007</td>
<td>0.006</td>
<td>0.006</td>
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<tr>
<td>(0.001-0.007)</td>
<td>(0.002-0.009)</td>
<td>(0.004-0.016)</td>
<td>(0.004-0.016)</td>
<td>(0.004-0.011)</td>
<td>(0.001-0.012)</td>
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</tr>
<tr>
<td>1,2,3,4,6,7,8-HpCDF</td>
<td>0.005</td>
<td>0.002</td>
<td>0.003</td>
<td>0.004</td>
<td>0.003</td>
<td>0.003</td>
</tr>
<tr>
<td>(0.002-0.010)</td>
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<td>(0.002-0.006)</td>
<td>(0.0024-0.0073)</td>
<td>(0.0017-0.004)</td>
<td>(0.001-0.0048)</td>
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<tr>
<td>OCDD</td>
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<td>0.00008</td>
<td>0.00012</td>
<td>0.00013</td>
<td>0.00007</td>
<td>0.0002</td>
</tr>
<tr>
<td>(0.00007-0.00090)</td>
<td>(0.00003-0.10500)</td>
<td>(0.00006-0.00020)</td>
<td>(0.00008-0.00020)</td>
<td>(0.00005-0.00010)</td>
<td>(0.00003-0.00028)</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Average results obtained by the EROD-H4IIE bioassay and by HRGC/HRMS.

Resultados promedio obtenidos por el bioensayo EROD-H4IIE y por HRGC/HRMS.

<table>
<thead>
<tr>
<th>Production Facility</th>
<th>HRGC/HRMS (pg WHO-TEQ/g fat)</th>
<th>H4IIE (pg TCDD-EQ/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>P1</td>
<td>0.24</td>
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</tr>
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<td>P3</td>
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</tr>
<tr>
<td>P5</td>
<td>0.22</td>
<td>0.09</td>
</tr>
<tr>
<td>P6</td>
<td>0.23</td>
<td>0.11</td>
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</tbody>
</table>
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Pork samples analysed by HRGC/HRMS, showed very low concentrations of PCDD/Fs and did not exceed the levels specified in domestic and foreign legislation (Minsalud 2008, EC 2006, Kim et al 2011). Only one sample was close to the intervention threshold for pork, established as a recommendation by the European Union (EC 2006, 2011). These results differ substantially from those detected in Chilean pork exported to the Republic of Korea and Japan in 2008, whose concentrations ranged between 2.17 and 36.7 pg WHO-TEQ/g fat, concluding that these animals were fed zinc oxide contaminated with PCDD/Fs as a premix ingredient (Kim et al 2011). It is

**DISCUSSION**

Figure 1. Comparison between results obtained by HRGC/HRMS in (pg WHO-TEQ/g fat) and H4IIE-EROD bioassay (TCDD-EQ pg/g tissue) in samples of pork.

Comparación entre resultados obtenidos mediante HRGC/HRMS (pg WHO-TEQ/g de grasa) y el bioensayo EROD-H4IIE (pg TCDD-EQ/g de tejido) en las muestras de carne de cerdo.

Figure 2. Correlation analysis between results obtained by EROD-H4IIE bioassay and HRGC-HRMS. There is no significant association between variables ($R^2 = 0.020$, $P \geq 0.284$).

Análisis de correlación entre los resultados obtenidos por el bioensayo EROD-H4IIE y mediante HRGC-HRMS. No hay asociación significativa entre las variables ($R^2 = 0.020$, $P \geq 0.284$).
therefore possible to infer that these contaminated pre-
mixes were added to the diets of pigs after the year 2007
and the negative sample for 2011 would indicate that mea-
ures taken by the Chilean pork industry to prevent anoth-
er case of contamination could be fulfilling their purpose.

The EROD-H4IIE bioassay for the detection of
PCDD/Fs has proved to be very useful, since it is fast,
reproducible, accurate, high sensitivity and lower cost
than HRCG/HRMS (Denison et al 2004, Whyte et al
2004) and has been used successfully in fish and wild-
life, among others (Whyte et al 2004). In this study the
bioassay lasted an average of 12 days from the beginning
of the extraction until the reporting of the results and it
showed a high sensitivity (LOD = 0.142 ± 0.1208) and a
high reproducibility considering the quality control crite-
ria of the assay.

Schoffer et al (2011) demonstrated the equivalence
between the bioassay and HRCG/HRMS for the detec-
tion of dioxins in broiler chicken meat, reporting that it
can be a useful technique to biomonitor these xenobiotics
in animal production. However, in this study it was not
possible to estimate a significant equivalence between
the results obtained from both methods in pork sam-
bles, because there was not significant correlation be-
 tween the data produced by the two analytical methods,
this precluded the use of a model to estimate the expec-
ted value of PCDD/Fs contamination, which would be
obtained by HRCG/HRMS, only if the EROD-H4IIE
bioassay is used. One of the problems identified is the
overestimation of the values measured by the bioassay,
regarding HRCG/HRMS. This observation was also re-
ported by Hoogenboom et al (2007) who applied the DR
CALUX® bioassay (using genetically modified H4IIE
cells) to analyse 219 samples, mostly from pork fat, with
80 of them being identified as suspicious and showing a
much higher response than the reference samples. The-
se samples were analysed by HRGC/HRMS, effectively
confirming 36 as positive. Overestimates have also been
reported in the analysis of poultry samples (Schoffer et
al 2011) and ash (Till et al 1997). The difference be-
tween the two methods may be due to compounds that
can trigger the aryl hydrocarbon receptor (AhR) pathway,
such as polycyclic aromatic hydrocarbons (Chaloupka et
al 1993), polybrominated compounds (Baston and De-
nison 2011), indol-3-carbinol (13C), which is present in
 cruciferous plants and has been described as a moderate
activity binder with the AhR receptor (Chen et al 1996),
in addition to DL-PCBs which in this study work were
not analysed by HRGC/HRMS. In this sense, it is neces-
sary to include a method of extraction and separation of
DL-PCBs from dioxins in the sample. On the other hand,
the underestimation of results by H4IIE, which limits the
bioassay, could be explained by the fact that some com-
pounds that induce the EROD/CYP1A1 activity are also
substrates for the enzyme CYP1A1, resulting in inhibi-
tion of response by competition that could result in an
underestimation of the total EROD activity obtained in
complex samples (Denison et al 2004). An example of
induction and inhibition has been described by Petrulis
et al (2001) in samples contaminated with mixtures of
halogenated aromatic compounds (HAC).

With regard to the positive samples detected by the
EROD-H4IIE bioassay, considering the maximum limi-
tation allowed under Chilean legislation (2 pg WHO-
TEQ/g fat), only 5 samples (8.5% of the total number of
samples) should have been confirmed by HRGC/HRMS;
the number would have risen to 15 (25.4% of the total)
if the European standard had been followed (1 pg WHO-
TEQ/g fat), therefore, with either option and if applying
monitoring to control the safety of meat with request of
PCDD/Fs, the savings would have been substantial com-
pared to the chromatographic analysis of the 59 samples.

Over 300 scientific studies have been published con-
cerning the use of EROD-H4IIE bioassay, highlighting
its characteristics of speed, simplicity and ability to inte-
grate the effects of complex mixtures, allowing it to be a
powerful analytical tool to be applied in risk assessments
and biomonitoring of dioxins and DL-PCBs, but extreme
care must be taken to generate reliable results (Whyte
et al 2004). Since the results of this study indicate that
the bioassay can be applied in pork for the detection of
dioxins, further studies are needed to achieve equivalence
between both methods, incorporating the removal of DL-
PCBs and following the strict application of a program of
quality control and quality assurance, including inter-
laboratory calibration tests.

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