Validation of rapid dioxin screening by GC-FID in fish products

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A novel, cost- and time-effective dioxin screening method was developed and validated for fish product. The method is based on multivariate covariance between fatty acid composition monitored by GC-FID and dioxin content as teq WHO pg/ g fat. A dioxin range varying from 1.1 to 47.1 pg TEQ-WHO/ g fat using 65 fish meal samples was accessible for model calibration. An optimal multivariate dioxin prediction model was developed based on automatic peak integration, thereby enabling extraction of the area of 140 peaks from the gas chromatogramms. Models were produced employing partial least squares regression (PLS) based upon the duplicate GC-FID run and 46 specific peaks, selected after variable selection from the 140 investigated. The best results were yielded by local pls modelling employing three latent variables based upon the 12 nearest neighbors. For each prediction sample, the neighbors, yielding the 12 smallest sum of squares of differences to the test sample using the 140 peaks, were extracted from the whole calibration set and a local model built using these 12 chromatograms and related dioxin content. Prediction performance was thereafter validated for 10 fully independent samples. The performance of this model, yielded a correlation of 0.85 (r^2) and a root mean square error of prediction of 2.3 pg PCDD/F TEQ-WHO/ g fat.

Introduction

The principal dioxin contamination in fish products is due to bioaccumulation through the food chain[1-3] implicating food, especially animal and fish products, as the main cause of background exposure. Generally, persistent lipophylic organic pollutants (PLOP's) such as polychlorinated dibenzo(p)dioxins and dibenzofurans (dioxins) and polychlorinated biphenyls (PCB's) accumulate in fat tissues, according to feeding habits and respective feed dioxin contamination. Accordingly, reliable, time- and cost-effective screening methods for dioxin content in food and feed are required for effective reduction of dioxin intake through nutritional pathways. Marine fats have been identified as a main source of dioxin with respect to human consumption[4-6]. HR-GC/GC-MS based methods are not compatible with highly desired at-line routine and extensive control, and alternatives are therefore investigated. Hence, the development of alternative monitoring methods for dioxin content in fish and related fish products. Four important types of alternative rapid

screening assessment have been recently presented: 1) Enzyme Immuno Assay (EIA) methods directed towards the most toxic of the 17 monitored dioxins, i.e., TCDD 2,3,7,8[7;8], 2) bioassay based on the Aryl Hydrocarbon Receptor (AhR) activation by PLOP's[9;10], 3) a promising method based upon fluorescence detection and advanced multivariate analyses (PARAFAC)[11], and 4) recently, a method, based on the same chemometric principles, this time employing fatty acid pattern recognition was proposed [12]. The focus of this paper is to validate this latest method based upon fatty acid pattern recognition for dioxin (TEQ- WHO) prediction in fish meal.

Material & Methods

Sample and sampling

Calibration set

In this study 65 fish meal samples were isolated to cover an important dioxin range. A first selection of 40 samples was gathered in February 2002, and a second selection of 25 samples in December 2002. The two sets spanned from February 2001 to November 2002, thereby covering a broad spectrum of raw material from herring and various fish trimmings, and whole fish such as sprat, blue whiting, capelin and sand eel. The catches were realised in the Baltic, North, Barents and Iceland seas, and were subjected to seasonal variations and available quotas. Each sample constituted 1.5 kg of fish meal isolated and mixed thoroughly prior to separation in three equal fractions: one fraction was sent for dioxin analysis by HR-GC/GC-MS at Eurofins Deutschland (GfA), the second was analysed for fat (by NIR) and fatty acid profile (GC-FID) at the fish meal plant and the third fraction was kept as a backup sample. This was carried out separately for the two independent sets. Finally the data from these two sets of fish meal were employed together as one calibration set.

Validation set

A fully independent validation set (a third set), consisting of ten additional samples selected in January 2004, was analysed following the same procedure as for the previous two sets employed for calibration.

Fat assessment

A precise estimate of fat content was obtained using NIR spectroscopy calibration by PLS (Paragon FT-NIR Identicheck and QUANT+ software vers. 4.51, Perkin Elmer, UK) updated monthly against soxlet resulting in a database representing approximately 800 samples ranging from 2.9 to 15.7 % fat w.w. and with an average of 10.0 % w.w.. By this procedure total fat content could be assessed in approximately one minute with an average prediction error, expressed as root mean square error of prediction, of 0.56 % w.w..

Reference analysis for dioxin assessment

Dioxins expressed in ng TEQ-PCDD/F /kg were monitored as the TCDD 2,3,7,8 relative toxicity weighted sum of polychlorinated dibenzofurans and dibenzo(p)dioxins (PCDD/F's), including limits of detection (LOD) and according to WHO. In short, samples were analysed for dioxin TEQ-WHO in Germany at GfA - Eurofins[13]. The solutions of the sample were cleaned up by liquid/solid chromatography after addition of sixteen $^{13}C_{12}$-labelled internal tetra- through octaCDD/F's standards. Prior to the gas chromatographic analysis, two further ^{13}C-labelled PCDD/F standards were added to the PCDD/F fraction for the determination of the recovery of the internal standards. A

capillary gas chromatograph (HRGC, HP 5890) coupled with a high resolution mass spectrometer (HRMS, VG-AutoSpec, mass resolution ≥ 8000) was used for the PCDD/F's analysis. The quantitative determination of native Tetra- through OctaCDD/Fs was achieved via the corresponding $^{13}C_{12}$-labelled internal standards (Isotope dilution method; GfA PA_156/97 DIN EN ISO/IEC 17025:2000 accredited method). On the basis of the PCDD/F concentrations and including LOD for the undetected PCDD/F congeners, TEQ-values according to the WHO model were calculated. The original measurements in ng TEQ- PCDD/F-WHO /kg w.w. product were converted to ng TEQ-PCDD/F -WHO /kg fat (pg TEQ-WHO / g fat), according to the respective fat content of the sample.

Fatty acid profile

The fatty acid composition was determined by Gas Chromatography (GC Autosystem XL, Totalchrom software, Perkin Elmer, UK) using a chrompack capillary column (wcot fused silica 25 m x 0.25 mm from Varian) and a Flame Ionization Detector (FID). Prior to injection, the oil included in the fish meal was direct-methylated: 0.7 g of fish meal sample was methylated by the addition of 1.5 mL toluene and 1.5 mL sodium methylate (3% v.v. sodium methylate in methanol) and incubated at 50°C for 10 min. After cooling to room temperature, 2.0 mL of ion exchanged water and 5.0 mL isooctane were added. After 30 min 0.5 mL of the supernatant (isooctane fraction) was transferred for drying in tubes containing 0.2 g of sodium sulfate. 40 µL of this solution was then transferred into vials and diluted with 1.8 mL of isooctane.

Finally, 1 µL of the methylated sample was injected to the GC in split-less mode by the autosampler and run for 35 min. The injector was set at 240°C. The oven temperature was initially set at 90°C with a first ramp of 30°C/min up to 150°C (0 min hold) and a second ramp of 3°C/min up to 225°C, with a 6 min hold. The detector was set at 270°C. Helium pressure was set at 22 psi, hydrogen flow was set at 45 mL/min and synthetic atmospheric gas flow was set at 450 mL/min (all gases were of grade alfagas type 1). Between samples the column was washed with isooctane. All analyses were run in duplicates. The measurements resulted in gas chromatograms reflecting fatty acids composition between C14:0 and C24:1n-9.

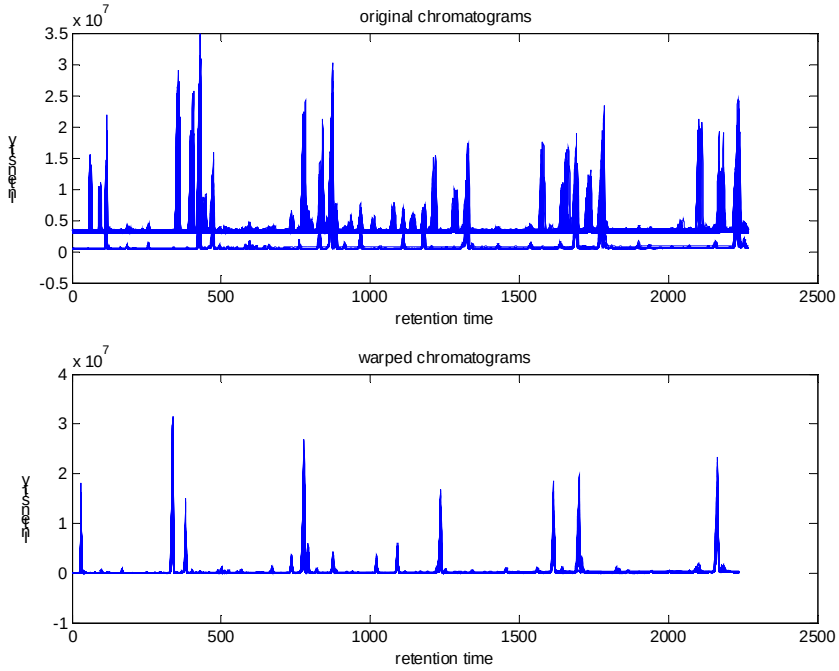

Fig. 1. Illustration of the 150 original and warped (time shift corrected) chromatograms obtained by GC-FID: the aligned data enable further multivariate data analyses.

Data processing

Warping (retention time correction):

Dynamic Time Warping [14] was performed to correct retention time shift. After removal of injection peak, the chromatogram dataset was reduced to 1:8, thereafter simple linear interpolation was employed between the first and last peak to stretch all the chromatograms to same length, and thereby supplying suitable data for warping. The warping procedure employed a segment of 25 pts and a slack of 1. 140 peaks were thereafter detected and integrated for peak area calculation resulting in 140 variables (independent variables).

Variable selection:

Variable selection was performed by stepwise backwards variable removal [15;16]. using backwards interval-PLS (bi-PLS) developed by R. Leardi and L. Nørgaard (in preparation), an extension of interval-PLS[17] was employed for this selection, by defining each interval as one variable (or peak). PLS models employing the calibration set (with three latent variables) for dioxin prediction were calculated leaving one variable out at a time, the model resulting in minimal Root Mean Square

Error of Cross Validation (RMSECV) was retained. This procedure was performed in an iterative manner, until variable removal induces RMSECV to increase again.

Multivariate calibration:
Partial least squares regression with three latent variables, based upon the 12 nearest neighbour selection [18] out of 130 chromatograms (duplicate GC-FID runs for each of the 65 samples) was performed employing Eigenvector Research Inc.'s Toolbox (vers. 3.00) for MatLab (vers. 6.5, MathWorks, Inc.). Thus, a new local calibration model was built for each sample to be predicted.

Results

Dioxin analysis

The fish meal samples presented a large dioxin range, varying from 0.13 to 4.89 with an average of 1.47 pg TEQ-WHO / g sample. The 75 samples were estimated by NIR to have a fat content varying from 8.1 to 13.4 % w.w., with an average of 9.6 % w.w. and a standard deviation of 1.25 % w.w.. When weighted in relation to the respective fat content of each of the fish meal samples, the dioxin range varied between 1.1 to 47.1 with an average of 14.4 pg TEQ-WHO / g fat. This large range of dioxin content was expected due to the raw material employed. Indeed fish meal samples were selected for this study with special attention to fishing zones, production period and fish species to cover realistic and representative production situations and with the intention to build up a calibration and a validation set with suitable large dioxin variation.

Fatty acid profile: gas chromatograms

Collected samples presented a broad variation in fatty acids (Fig. 1) reflecting seasons, locations and species. However, traceability down to single species was not reached, fish meal processing relying on the mixture of available catches.

Multivariate analyses

Partial least squares regression using the 12 nearest neighbours confirmed the dioxin and fatty acid profile relationship. As presented in Table 1, valid predictions of dioxin from the full fatty acid pattern were obtained. Partial least squares regression using three latent variables (PLS components) resulted in a correlation r^2 of 0.89, and a Root Mean Square Error of Prediction (RMSEP) of 3.6 pg TEQ-WHO / g sample fat. The calibration error estimated as Root Mean Square Error of Cross Validation (RMSCV) was down to 1.1 pg TEQ-WHO / g sample fat, illustrating a potential overfit when compared to the RMSEP value of 3.6 obtained on the independent test set. In contrast the multivariate model was significantly improved by variable selection. The 13 selected variables resulted in similar correlations. The model based upon reduced data yielded a higher RMSECV (2.1 pg TEQ-WHO / g sample fat), agreeing with an optimal RMSEP now reduced to 2.3 pg TEQ-WHO / g fat (Table 1). Taking into account the range of dioxin covered by the validation sample from 2.3 to 18.7 and the related average of 8.6 pg TEQ-WHO / g fat, the error of prediction, RMSEP of 2.3 pg TEQ-WHO / g fat, can also be expressed as 14 % interval scaled or as 27 % average scaled (CV). Moreover the repeatability of the reference measurement for a sample of 8.4 pg TEQ-WHO / g fat yielded a standard deviation of 1.1 pg TEQ-WHO / g fat and consequently a CV of 13 %. The variance being additive, the error due to the reference measurement can be estimated to be $\geq$ 1.1 for the considered range. Correcting the

apparent RMSEP of 2.3 from the reference error, the corrected RMSEP can then be estimated to be 2.0 pg TEQ-WHO / g fat or 12 % as interval scaled and 23 % as average scaled (CV)**.**

Table 1. PLS calibartion and validation with three latent variables (LV) for dioxin (as pg TEQ-WHO / g fat) prediction models based upon fatty acid pattern recognition, centred according to the 12 nearest neighbour and according to selected variables.

Calibration set	**Validation Set**	**Selected variables**	**Total dioxin range**	**LV**	**Calibra -tion (r^2)**	**RMSECV** dioxin pg / g fat	**Valida -tion (r^2)**	**RMSEP** dioxin pg / g fat
65 samples	10 samples	140 (all)	1.1-47.1	3	0.99	1.1	0.89	3.6
65 samples	10 samples	46	1.1-47.1	3	0.96	2.1	0.85	2.3

Discussion

The present work illustrates how fatty acid profiles can be employed for dioxin (TEQ-WHO) screening (Fig. 2 and Table 1). The presented data confirmed previous studies illustrating potential covariance between plasma omega-3 polyunsaturated fatty acids and blood dioxin and dioxin-like content in relation to fish intake[5;6] in contrast to other PCB studies[19]. In these earlier studies, human alimentary habits were illustrated by plasma omega 3 fatty acids and related to PLOP's. We have previously demonstrated that a similar relationship exists between TEQ-WHO dioxin and a specific group of fatty acids in fish meal (Bassompierre *et al.*, in press) and emitted following hypothesis for the strong quantitative relationship presented here between the fatty acid pattern and the bioaccumulation of dioxins: direct relationship through metabolic pathways due to oxidative stress mechanisms[20], b) species related characteristics[21] or c) accumulation along the alimentary chain according to the fish feeding trophic level pyramid[4-6;22], d) mixing effects according to fish trimmings availability. Independently of the exact underlying causes, fatty acid patterns and dioxin TEQ WHO contents are both results of historical events where some systematic and parallel evolution, dependent or independent have led to the illustrated relationship. And we are hereby successfully validating the dioxin screening method based upon fatty acid pattern recognition by GC-FID with an independent test set. Moreover, the presented method is conforming to EU requirements for dioxin screening (directive 2002/70/CE), CV being required to be below 30 % for screening methods.

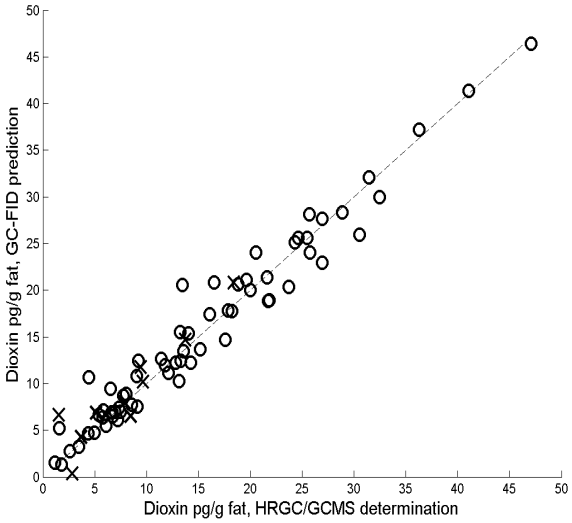

Fig. 3 Predicted dioxin values versus reference HR-GC/GC-MS TEQ-WHO measurements. The predictions were obtained by PLS calibration model employing nearest neighbour centring including 65 fish meal samples (o) and based on the 13 selected fatty acids. The samples constituting the validation set are plotted with x.

Conclusion

The presented dioxin screening method based on pattern recognition of fatty acid profiles perform remarkably well for fish products such as fish meal, and the validation of the prediction performances confirms previous publication[12]. The implementation of the method should be simple and cost effective: requiring only basic chemometric software and fatty acid profile determination. The dataset and related calibration providing the core of this dioxin screening method required only fatty acid determination typically performed by direct methylation followed by GC-FID. Some fish and fish-related industries already have fatty acid assessment facilities and will easily be able to implement this dioxin screening method, thereby accessing, in a cost effective manner, in house answer with an analysis time improved by factor 1000! Others will have to purchase fatty acid profiles performed by commercial laboratories.

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