**Main S&T results/foregrounds**

**Developing detection methods for organic pollutants; POPs, PFCs, Pesticides**

**WP1a Persistent Organic Pollutants**

**Achievement 1a-1: Integrated sample preparation.**
The integrated sample preparation strategy for the determination of key representatives of polychlorinated biphenyls (PCBs), brominated flame retardants (BFRs) and polycyclic aromatic hydrocarbons (PAHs) in fish was developed, successfully validated and finally the manuscript documenting this work was published by Kalachova et al. in 2011 in Analytica Chimica Acta. The sample preparation is based on ethyl acetate extraction of wetted matrix realized by 1 min shaking, the transfer of analytes into the organic layer was supported by added inorganic salts. Further clean-up is carried out on silica SPE minicolumns and final instrumental analysis is achieved using gas chromatography coupled to time of flight mass spectrometry (GC–TOFMS).

Applying this integrated sample preparation, significant time, workload and cost savings can be achieved. This was one of the main reasons why the CONffIDENCE method was selected for international validation by AOAC. Recently, it has been confirmed as ‘official method’ for analysis of PAHs in fish and seafood.

**Achievement 1a-2: GC–MS method.**
Two dimensional gas chromatography coupled to time of flight mass spectrometry (GC×GC–TOFMS) was optimized and validated to provide the best separation of POPs and, concurrently, low limits of quantification (LOQs) to fulfill the legislative requirements. A method for the simultaneous identification and quantification of all 41 analytes including PCBs, BFRs and PAHs was obtained and LOQs in the range of 0.01–5 µg/kg were achieved. The method was published by Kalachova et al. in 2012 in Analytical Bioanalytical Chemistry.

Additionally to GC×GC–TOFMS, an alternative GC–MS method for the determination of a wide range of BFRs was implemented, also including emerging compounds which were not on the target list of the CONffIDENCE project. For this purpose, GC coupled to tandem mass spectrometry (GC–MS/MS) with a triple quadrupole ion analyser was optimized, resulting in distinctly improved selectivity and sensitivity compared to routinely used single quadrupole MS. Accurate determination of (ultra)trace levels of BFRs, which might be of concern under certain conditions, e.g. within total diet studies, was achieved. The quantification limits were
Achievement 1a-3: Screening POPs in fish.
A multiplex imaging immunoassay (IMIA) for selected POPs (PCB 77, PBDE 47 and BaP) was developed using superparamagnetic spectrally encoded microspheres (SEMs) and a new imaging platform with a planar readout. The performance of the 3-plex IMIA in buffer and tilapia extracts was critically compared to the previously developed 3-plex flow cytometric immunoassay (FCIA) and found to be similar. A preliminary in-house validation with 40 different tilapia fillet samples, blank and spiked with different POPs at relevant levels, was performed in both assay platforms. The outcome of the pre-validation study demonstrated the high potential of both 3-plex immunoassays to screen for POPs in tilapia fillet at half of the ML for PCBs and at relevant levels for PAHs and the emerging PBDEs. The 3-plex IMIA has the clear advantage of a relatively low cost and easy transportable system. After further application and validation in a range of different matrices, it can be a useful pre-screening tool for POPs in fish and possibly in other environmental samples.

Achievement 1a-4: Monitoring survey.
In a close cooperation with WP1b – dealing with perfluorinated compounds and WP3 – dealing with heavy metals, a joint survey focused on the occurrence of these different groups of pollutants in fish samples was realized. Within WP1a, not only halogenated pollutants, but also the fat content and the profile of polyunsaturated fatty acids (PUFAs) was determined as a contribution to risk-benefit models of POPs and PUFAs occurring in fish. In order to analyse different European diets through the fish available on the market, the sampling strategy was focused on various fishing areas represented by Mediterranean Sea (Spanish coast), Cantabric Sea, North East of Atlantic Ocean, North Sea and Baltic Sea. Altogether 140 samples were analysed including 18 different species (bivalves, whiting, cod, hake, herring, salmon, trout, tuna from European waters, as well as pangasius fish from Vietnam) both of wild and aquaculture origin.

Three different POP groups were monitored using the previously developed method which enables their simultaneous determination: (i) non-dioxin like (NDL) PCBs (indicator congeners 28, 52, 101, 138, 153 and 180) and dioxin-like (DL) PCBs (non-ortho congeners
77, 81, 126 and 169 and mono-ortho congeners 105, 114, 118, 123, 156, 157, 167 and 189); (ii) 16 PAHs (only the content of PAH4 – BaA, BaP, BbFA and CHR is regulated in food) and (iii) PBDEs (congeners 28, 47, 99, 100, 154, 153 and 183).

As expected, from the PCB groups, the highest levels were measured for NDL PCB congeners, mainly for CB-153, CB-138 and CB-180. Their levels were 5-10 times higher compared to mono-ortho congeners and appr. 100-times higher than those determined for non-ortho PCBs. From DL-PCB congeners, pentachlorinated CB-105 and CB-118 were detected at highest levels but none of the samples exceeded the legislative limits. From the PBDE group, congeners BDE-47, BDE-99 and BDE-100 were the most abundant with levels appr. 10-times lower compared to NDL-PCB and comparable to mono-ortho PCBs. As regards PAHs levels in fish, they were also very low, only approaching LOQs, while bivalves were the most contaminated samples by PAHs.

When comparing the contamination of individual fish species, in case of halogenated POPs, the contamination extent can be classified in following order: herring ≈ whiting > salmon ≈ trout > bivalves > hake > pangasius ≈ tuna ≈ cod. Regarding PAHs, as expected, bivalves were much more contaminated than fish since bivalves do not metabolize PAHs. When comparing the different regions of origin, fish samples from the Baltic Sea were the most contaminated. Nevertheless, none of the examined fish/bivalves samples exceed legislative limits for any of the regulated contaminant groups.

**Achievement 1a-5: DART-MS lipids profiling.**

A new innovative analytical technique based on ambient mass spectrometry employing Direct Analysis in Real Time (DART)-MS for characterization of fish samples, mainly of the lipid fraction including triacylglycerols (TAGs) and fatty acids (FAs), was developed. The main advantage of this new technique for assessment of fish lipids quality is the possibility to measure the metabolomics (lipidomic) profile in the same ethylacetate extract which is examined for levels of organic pollutants. No saponification of TAGs is needed and the DART-MS measurement only takes several seconds. Characteristic mass spectra (metabolomic fingerprints) are obtained, enabling identification and authentication of individual species.

The fingerprints (mass spectra corresponding to both negative and positive ions) obtained on various fish species (herring, hake, tuna, whiting, cod, trout, salmon and pangasius) and bivalves were compared. While in most marine fish species TAGs with higher m/z value i.e. those containing ‘long’ PUFA such as arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid were present, these TAGs were absent in pangasius fish, which indicates a lower nutritional value.
WP1b Perfluorinated Compounds

Achievement 1b-1: The development of simplified analytical methods based on liquid chromatography coupled to mass spectrometry (LC-MS) for the analysis of perfluoroalkyl substances (PFAS) in food and feed.

An innovative and simplified protocol for the analysis of the three selected PFASs (PFOA, PFOS and FOSA) in food and feed samples was developed, based on methanol extraction followed by clean-up by means of dispersive solid phase extraction with activated charcoal and, finally, detection by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). The methodology was adapted for the extraction of PFASs from fish, fish feed and milk (Figure 1b-1-1).

The work was published in the journal Proceeding of the 6th International Students Conference - Modern Analytical Chemistry and the results of the validation can be found in the Czech Journal of Food Science. As an example, a flowchart of the extraction of PFASs from seafood is given in Figure 1b-1-2.
Achievement 1b-2: The validation, harmonization and verification of these analytical methods using a step by step approach including the organization of interlaboratory studies at European level.

Two different levels should be differentiated in this section. The first level corresponds to the validation in-house and method transferability between WP1b partners. The second level corresponds to the performance of two different interlaboratory studies for the assessment of the methodology for the extraction of PFASs from fish, fish feed and milk.

Method optimization

The method validation was carried out in 2010 for both matrices (milk and fish) following the 2002/657/EC decision guidelines. According to the quality parameters, both methodologies (for fish/fish feed and milk) are suitable for the analysis of PFASs in the matrices of interest. These methods are reproducible, robust, sensitive and fast. Once the methods were validated by the same laboratory that developed the procedures, the method transferability was evaluated by the other WP1b partners. From the transferability study it was concluded that the methodologies are suitable for the analysis of fish/fish feed and milk samples.

Collaborative studies
Two collaborative studies for the analysis of PFASs in fish, fish feed and milk have been performed among expert analytical laboratories from different European countries. From the second collaborative study, which was a full collaborative study with 8 participating laboratories, it was concluded that the developed methodology can be applied in routine laboratory analysis for PFASs determination in milk, fish feed and fish at levels down to 1 µg/L or 1 µg/Kg, respectively. The transfer of the developed method to other laboratories was successful, which was demonstrated by the satisfactory results, particularly for fish and fish feed. The related journal papers will be submitted in the ABC special issue.

**Achievement 1b-3:** The analysis of real food samples by the developed methodology in order to contribute in the [European PFASs data base](#) and the main inputs through the diet.

This achievement was performed by the analysis of most consumed sea fish and bivalves from Europe and popular farmed fish. Additionally, a small scale study of Mediterranean dairy products was performed. For the sea fish and bivalve study, different European markets from regions with different dietary habits were selected including samples from Baltic Sea, North Sea, Atlantic Ocean and Mediterranean Sea as well as some river samples. For sampling purposes, four different institutes, all of them partners of the CONffIDENCE project, cooperated during the sampling process into the different markets: ICT (Prague), DTU (Denmark), RIKILT (The Netherlands) and IDAEA-CSIC (Barcelona) (Figure 1b-3). A total number of 124 samples were analyzed including bivalves, farmed salmon, hake, cod, whiting, herring, trout, tuna and farmed pangasius (this last from Vietnam fishing area).

In order to approximate the levels found to a real European diet through the ingestion of fish, the Daily Intake (DI) for PFOA and PFOS was calculated. The DI was evaluated for a man and woman between 25 and 40 years with a medium weight (70 and 60 Kg, respectively). The proposed diet, among other food that can be taken, was the consumption of 300 g of fish fillet. The DI was calculated according to: $DI \text{ (ng/Kg day)} = [(\text{Consumption x PFC concentration}) / \text{ body weight}].$ The risk index (RI) was calculated, as in [previous published work](#) by the same authors: $RI = [DI / TDI],$ where TDI is the Total/Tolerable Daily Intake (150 ng/Kg day for PFOS and 1500 ng/Kg day for PFOA, taken form EFSA (2008)). The obtained RI for PFOS and PFOA demonstrated that no immediate risk for human health through the consumption of the investigated fish and bivalves is expected.
**Achievement 1b-4:** The evaluation of PFASs toxicity using standardized methods.

Toxicity assessment of PFOS, PFOA and FOSA was carried out according to the method based on the bioluminescence inhibition of the standard organism *Vibrio fischeri* originally described by Bulich. The toxicity of the standard individual PFASs was measured over a wide range of concentrations in order to plot a well defined inhibition curve. The 50% effective concentration of each substance (EC50) was calculated. The toxicity units (TU), defined by Sprange and Ramsay equation, were calculated as: \[ TUs = (EC50)^{-1} \times 100. \] Figure 1b-4 presents inhibition curves for PFOS, PFOA, and FOSA vs. *Vibrio fischeri*. A luminometer that was designed in full agreement with the DIN/ISO bioluminescence inhibition protocol for toxicity assessment was used to measure the light inhibition during the experiment. Incubations were carried out at a controlled temperature (15 °C) and the incubation times were 15 min and 30 min.

The results showed that these compounds have low acute toxicity levels. Toxic effects due to chronic exposure have not been tested in the current research, but it is recommended to consider these due to the chronic nature of the exposure to these chemicals.

![Inhibition curves after 30 min. exposure](image)

**Figure 1b-4:** Inhibition curves for *Vibrio fischeri* bioluminescence assay at 30 min of exposure
WP1c Pesticides

**Achievement 1c-1: Direct detection of Dithiocarbamates in fruit/vegetables by Ambient MS.**

Dithiocarbamates are widely used fungicides which are normally analysed through their common hydrolysis product CS\(_2\). In CON/IDENCE the feasibility of **rapid detection of intact thiram and ziram from fruits and vegetables through ambient mass spectrometry** (DART and DESI) has been demonstrated. This enables compliance testing of products with specific maximum residue limits that have been established in the EU for these dithiocarbamates.

**Achievement 1c-2: Detection of paraquat in potatoes/cereals by electrochemical immunosensor.**

Paraquat is a broad spectrum herbicide that is also used as desiccant to facilitate in the harvest of certain crops. Since 2007 this product has been banned in the EU but it is still widely used outside the EU. Paraquat is not amenable to chemical multi-residue methods and rapid screening methods would be highly beneficial for residue monitoring. A new electrochemical immunosensor has been developed. Paraquat is measured through antibodies labeled with semiconductor nanoparticles which are electrochemically detected. Although the primary target analyte was paraquat, the assay has been demonstrated to be suited for multiplex detection by using different types of nanoparticles using deoxynivalenol as a model second target. The assay is extremely sensitive for paraquat. Even when diluting the potato and cereals extracts 100-fold, detection limits of 1 µg/kg can be achieved. The sensor was successfully validated for compliance testing against the EU-MRL (0.02 mg/kg).

![Figure 1c-1: DESI/DART method](image1)

![Figure 1c-2: Electrochemical sensor](image2)

**Achievement 1c-3: Survey for paraquat and diquat.**

For paraquat and the related herbicide/desiccant diquat, very few data on occurrence of residues is publicly available so far. A survey was conducted to gain insight in the occurrence of residues of paraquat and diquat in food and feed commodities. In order to include diquat in the survey, LC-MS/MS was used for detection. A simplified extraction
procedure for this method was developed within the CONffIDENCE project. Almost 300 samples were analyzed, including potatoes, cereals, dry pulses and other food/feed commodities. Paraquat was not detected in any of the samples while diquat was detected in 5% of the samples in the range 0.005-0.11 mg/kg. Residue-containing crops were lentils, potatoes, barley and oilseed meal. Organic products were included in the survey. One batch of lentils labeled as organic contained diquat. None of the residues exceeded the EU-MRL.

Figure 1c-3: Samples analyzed for the survey

Developing detection methods for Veterinary Pharmaceuticals: Coccidiostats, Antibiotics

WP2a Coccidiostats

Achievement 2a-1: New relatively fast and inexpensive multiplex method for screening of coccidiostats.
A multiplex assay for the detection of residues of lasalocid A, monensin, salinomycin/narasin, nicarbazin and diclazuril (the latter only in eggs) in laying hens feed and in eggs was successfully developed and single-laboratory validated. Details related to the development of the method were published in 2012 by Bienenmann-Ploum et al. in Analytical and Bioanalytical Chemistry. The assay involves a generic, simple prior extraction procedure and proved to be fit for the purpose of the determination of these residues at the maximum levels set in the latest EU legislation. Additionally, a comparison of the method performance characteristics of the new assay versus existing reference methods confirmed the fitness for screening purposes.

Fig 2a-1: New screening method
Achievement 2a-2: Inter-laboratory comparison.
The new multiplex assay was further and fully validated through a small-scale inter-laboratory comparison. Validation was achieved for the 6 coccidiostats in eggs and for all coccidiostats except diclazuril in feed. The inter-laboratory comparison allowed to establish robust cut-off values for all analytes in the multiplex assay. The rate of false positives in the blanks showed to be low enough to allow the use of the screening test, hence demonstrating the analytical fitness for purpose of the multiplex assay. Finally, for DNC (nicarbazin) in feed and diclazuril in eggs, the inter-laboratory comparison showed that the final decision for use of the screening test should be based on the cost situation and expected frequency distribution of these target analytes.

Figure 2a-2: Robust cut-off for monensin (Top: eggs; bottom: feed; cut-off: red line)

Achievement 2a-3: Carry-over study from feed to eggs.
Since no publication was available to provide us with the indications and parameters, such as partition coefficients, the compartment volumes, dose absorbed over the gut wall needed to build a modelling approach such as the PBPK model it was not possible to use this approach. In a feeding trial it was demonstrated that the relative incidence of lasalocid to accumulate in eggs expressed in ng g⁻¹ can be estimated using the range between 58 and 70 multiplied by the feed concentration (mg kg⁻¹). Therefore, the correlation between the lasalocid concentration in feed and the lasalocid residue concentration in egg has been confirmed applying a linear equation. This approach exhibits the major advantage of being rapid and quite reliable.
WP2b Antibiotics

Achievement 2b-1: Tetrasensor® for feed, urine and cooked meat
The Tetrasensor® is a competitive receptor based lateral flow dipstick assay developed by Unisensor that can detect many tetracycline compounds in different matrices such as milk, honey and raw animal tissues. During the CON//IDENCE project, the application of this kit was extended to feed, urine (http://www.unisensor.be/en/catalog/feed-31/tetrasensor-52.php) and cooked meat. After assay optimisation, a single laboratory validation (CD 2002/657/EC) was performed. This rapid, sensitive and easy to use test is capable of the detection of tetracycline compounds in a range of matrices below the required detection limit of 100 µg kg⁻¹, so that 50+ samples can be analysed in a day (up to 16 samples per h). The results were presented in poster format at ASSET 2011, RAFA 2011 and Euroresidue VII.

Achievement 2b-2: Bee4sensor® for honey (lab format and field test)
The Bee4sensor® is an indirect competitive immunochromatographic antibody-based dipstick assay for the rapid and simultaneous detection of sulfonamides, (fluoro)quinolones, tylosin A and chloramphenicol in honey that was developed within the CON//IDENCE project. The kit will be available from Unisensor (http://www.unisensor.be/en/catalog/antibiotics-28/bee4sensor-45.php) in (i) a lab-based format, undergone a single laboratory validation (CD 2002/657/EC) and a small-scale inter-laboratory validation (ILV) with 7 European laboratories, and (ii) a field test format, undergone a ‘proof of principle’ validation and global
field trial with 16 participants from across governmental, industrial organizations and academia. Herewith a rapid test for industry and enforcement authorities has been presented. The results of the successful ILV will be published in the special issue of the ABC journal; while the outcome of the single laboratory validation (lab-based format) and the excellent performance of the Bee4sensor® during the field trial will be reported at a later date.

Developing detection methods for Heavy Metals

WP3 Heavy Metals

Achievement 3-1: Inorganic As detection in seafood and rice by SPE-HGAAS and collaborative study.
A novel speciation approach has been developed in the CONffIDENCE project, which allows for the specific determination of inorganic arsenic by solid phase extraction (SPE), which separates inorganic arsenic from organoarsenic compounds, followed by detection by hydride generation atomic absorption spectrometry (HGAAS). The inorganic arsenic was extracted by use of microwave technology at 90°C. In a recent publication in ABC the method development, validation and application to a range of seafood samples has been described (Rasmussen, 2012). This novel separation approach was furthermore tailored for the analysis of rice samples, a sample type, which recently has drawn much attention with regards to its potential high levels of inorganic arsenic and hence the potential high contribution from rice to the dietary exposure of inorganic arsenic (EFSA, 2009). In this approach a simplified extraction procedure was developed by using a water bath and dilute acid, which allowed the simultaneous extraction of many samples. The method was applied to a range of rice samples and rice crackers and the results will be presented in an upcoming publication in the CONffIDENCE special issue of ABC (Rasmussen et al, 2013a). Both sample types (seafood and rice) were successfully tested in collaborative studies following a training session with the participating laboratories (Figure 3-1).

Figure 3-1: Participants at the inorganic arsenic workshop at the regional laboratory in Oldenburg, Germany

Achievement 3-2: Methyl Mercury detection in seafood by HPLC-ICPMS and collaborative study.
A method for the specific determination of methylmercury in marine food and feed samples was developed. The method procedure included a simplified extraction step with ultrasonification, which allowed for fast extraction of many samples at the same time. The
The concentration of methylmercury could subsequently be determined in the extracts by a HPLC-ICPMS procedure, where inorganic mercury and methylmercury were separated on a cation-exchange column followed by mercury-specific determination by ICPMS. The method was in-house validated and furthermore tested in three other laboratories on both feed and food samples. The results will be presented in an upcoming publication in the CONffIDENCE special issue of ABC (Rasmussen et al, 2013b). The method was also used to provide data on the methylmercury content (together with other pollutants) in fish feed and fish feed ingredient samples (Granby et al, 2013, to be published). The method addressed the need for speciation methods and speciation data specifically on methylmercury as recently emphasized by EFSA (2012) and JECFA (2011).

**Achievement 3-3: Survey on seafood.**

The developed methods were used in a large survey on seafood, which was undertaken in close collaboration with WP1a and WP1b. Data were collected on inorganic arsenic and methylmercury in a range of seafood samples, including lean and fatty fish and bivalves from various important fishing regions throughout the world. The dataset has provided valuable input for a better understanding of contaminant levels in seafood and will provide an important contribution to risk-benefit assessments of dietary seafood intake in the EU population (Figure 3-3).

![Figure 3-3: Methyl mercury in fish and fish feed](image_url)

**Developing detection methods for Biotoxins: Alkaloids, Marine Biotoxins, Mycotoxins**

**WP4a Alkaloids**

**Achievement 4a-1: Dipstick methods.**

Dipstick methods were developed for the tropane alkaloids scopolamine and hyoscyamine and for the ergot alkaloids ergometrine, ergotamine and ergocristine and were extensively tested and validated in in-house studies and in interlaboratory studies involving 7 participants. For both dipsticks the in-house validations were performed. The tropane alkaloid dipstick performed successfully at the target level of 800 µg/kg for the mixture of L-hyoscyamine and
L-scopolamine. The ergot alkaloid dipstick performed successfully at the target level of 200 µg/kg (for individual alkaloids) for ergotamine, ergocristine and ergometrine. Evaluation of the results of the interlaboratory study of the tropane alkaloid dipstick showed very satisfactory results, i.e. there was excellent separation between blank samples and samples containing the tropane alkaloids at 50% and 100% of the target levels. This makes the test quite suitable for screening purposes, since > 95 % of field samples are blank. It was noticed, however, that there exists a significant difference between the cut-off levels derived from the in-house and the interlaboratory studies, which could not yet be explained, and which needs further study. Preliminary evaluation of the results of the interlaboratory study of the ergot alkaloid dipstick also showed very satisfactory results. In this case there was no significant difference between the cut-off levels derived from the in-house and the interlaboratory studies. Regarding the practical use of the ergot alkaloids dipstick it is difficult to draw pertinent conclusions, because ergotamine and ergocristine are detected with different sensitivity on the same test line. This makes it difficult to calculate a combined and realistic cut-off level. Whereas not all relevant ergot alkaloids that can be present in rye ergot are detected, the 3 selected compounds represent a substantial portion of the total ergot alkaloids, normally present in ergot. For both dipstick assays results can be obtained within 30 minutes, and over 60 samples can be analysed per day per person. The method for the determination of the tropane alkaloids will be submitted for the special issue of the ABC journal. The method for the determination of the ergot alkaloids is proposed to be submitted for publication in a special issue of World Mycotoxin Journal about “Rapid Methods for Mycotoxins”.

Figure 4a-1: Dipstick procedure and reading for tropane and ergot alkaloids.

Achievement 4a-2: Multiplex ELISA.
A rapid multiplex method was developed to detect representatives of three important groups of pyrrolizidine alkaloids (senecionine, lycopsamine and heliotrine types) to be used as a screening tool for the detection of these toxic compounds in food and feed. The method was in-house validated for honey and feed matrices and was demonstrated to have a detection capability less than 25 µg/kg (for individual alkaloids) for jacobine, lycopsamine, heliotrine and senecionine. A reduction step was introduced to the extraction procedure to allow for the additional detection of the presence of N-oxides of PAs. With the developed method results can be obtained within 2 hours, and over 50 samples can be analysed per day per person. It is a first assay of its kind for PAs.

A small scale inter-laboratory study was set up to verify the trans-laboratory performance of the developed multiplex assay for the detection of the key PA: senecionine, heliotrine and lycopsamine. The evaluation revealed that for all 3 laboratories acceptable results for the rate of false-positives was obtained, with the exception of the results of 1 laboratory (for senecionine). Because in the inter-laboratory study only three laboratories could participate, it was deemed that a higher number of samples should be analysed to demonstrate the transferability of the assay. The method has been submitted for publication in the special issue of the ABC journal.

![Figure 4a-2: Multiplex ELISA for key pyrrolizidine alkaloids](image)

**Achievement 4a-3: NIR method.**

The protocol and validation of the NIR hyperspectral imaging method for the detection of ergot contamination in cereals dedicated for food and feed has been described in detail and published ([Food Additives and Contaminants, 2012](#)). The hyperspectral imaging method has been successfully rounded off with testing and demonstrating the system at an industrial site (see figure opposite). Relevant presentation and demonstration about this methodology has been presented at the dedicated CONffIDENCE Cluster workshop at WMFmeetsIUPAC, Rotterdam, the Netherlands in November 2012. It will be published in the special issue of the ABC journal. This technology can be used to detect and quantify ergot contamination in large samples of cereals destined for food or feed in both the laboratory and in the processing industry, where accurate and fast inspection is needed. The classical microscopy method can be used as confirmatory method on reduced samples. The line scan NIR hyperspectral imaging system allows to analyse a sample of 250 g in one minute. In comparison, the existing microscopy method requires 30-60 minutes to analyse the same sample.
WP4b Marine Biotoxins

Achievement 4b-1: Contribution to EFSA opinions on emerging marine toxins.
This Work Package was primarily concerned with conducting the research on a range of regulated and emerging marine biotoxins. With regards the emerging toxins spirolides and palytoxin; the focus was determining their modes of action so as to assist the European Commission in determining the potential risks of human exposure to these algal metabolites via ingestion of contaminated shellfish. The research conducted at the University of Santiago de Compostela in Spain provided highly important information which was published in the scientific literature and used by the European Food Safety Authority (EFSA) in producing their scientific opinions on these toxins. EFSA published these opinions in their own journal (2009. 7(12); 1393 and 2010. 8(6) 1628).

Achievement 4b-2: Multiplex biosensor assays for multiple shellfish toxins developed and validated.
The second major task for WP4b was to develop biosensor based tests for a wide range of regulated and emerging biotoxins. Antibodies to the major classes of regulated toxins, Paralytic Shellfish Poisons, Diarrhoeic Shellfish Poisons and Amnesiac Shellfish Poisons along with the emerging biotoxin palytoxin were utilized on a number of different biosensor platforms to determine if the testing for all compounds could be undertaken simultaneously. In order to do this a substantial amount of research had to be undertaken on developing novel surface chemistries and sample preparation techniques. The results of this research were that the multiplexed analysis of marine toxins was demonstrated for the first time and could be
applied to samples to rapidly detect toxin present in samples harvested from coastlines (McNamee et al, Environmental Science and Pollution Research. 2013, in press).

A biosensor assay was also developed and validated for the emerging marine biotoxin tetrodotoxin. The assay based on surface plasmon resonance was shown to be highly effective in detecting the toxin in a range of biological samples (Campbell et al, Special issue of the ABC journal, under review).

![Figure 4b-2: The prototype multichannel surface plasmon resonance biosensor used in the CONffIDENCE project](image)

**Figure 4b-2: The prototype multichannel surface plasmon resonance biosensor used in the CONffIDENCE project**

**WP4c Mycotoxins**

**Achievement 4c-1: Multiplex dipstick tests for fusarium toxins: 4 mycosensor**

The aim of mycotoxin research within CONffIDENCE project was to develop multiplex dipstick tests for the determination of the *Fusarium* toxins DON, ZEA, T-2/HT-2 and FBs in cereals and cereal products.

Prototype multiplex dipsticks were designed and assembled in order to reach detection limits (cut off levels) compatible with the EU legislation in force or expected for Fusarium toxins (EC Regulations No 1881/2006 and 1126/2007). Simplified sample preparation protocols were developed requiring no more than 6 min extraction with a mixture of methanol/water. The immunoassay kit, based on indirect competitive format, is composed by multiplex dipsticks, a set of microwells containing freeze-dried reagents, and an incubator. Results are interpreted by an optical reader measuring the ratio between each test line and a dynamic control line located on the top of the strip. The resulting immunoassay protocol is rapid (total analysis time 50 min for 6 mycotoxins), inexpensive, and easy-to-use.

The kit is now commercially available and distributed by Unisensor, that was active partner in the project.
Achievement 4c-2: Interlaboratory validation

A “single-laboratory validation design” was elaborated and applied to evaluate performances of multiplex dipstick immunoassays for the determination of *Fusarium* toxins in wheat and maize. Statistical evaluation of the results (ANOVA) showed the ruggedness of the assays, since neither the matrix (i.e. wheat/maize origin) nor the day effect inflated the variation of the results. The rate of false positives was generally lower than 10% for all toxins. Results of single-laboratory validation will be published in the special issue of the ABC journal.

The test was subjected to final validation by a full collaborative study involving 13 laboratories. A training phase was included in the experimental design enabling the participants to familiarize with the protocol. The total standard deviation of the response varied from 6 to 24% for the analyte/concentration/matrix combinations included in the study, indicating the assay ruggedness between different laboratories, and therefore, the method transferability. A manuscript describing results and outcomes of the interlaboratory study is in preparation for the special issue of the ABC journal.

Finally, to evaluate the fitness-for-purpose of the test, the performance profile was applied to realistic distributions of the mycotoxins in target materials obtained from European monitoring programmes.

A practical training on determination of *Fusarium* toxins in maize by multiplex dipstick immunoassays developed in CONffIDENCE project was organized within the ISM-MycoRed Training Course “Detection techniques for mycotoxins and toxigenic fungi in the food chain”, May 28 – June 1, 2012. ISPA-CNR, Bari, Italy.

Furthermore the commercial kit has been presented at the dedicated CONffIDENCE Cluster workshop at WMFmeetsIUPAC, Rotterdam, the Netherlands in November 2012.

Potential impact, main dissemination activities and exploitation of results

The safety and quality of food and feed are a growing public concern and research plays an increasingly important role in this sector to ensure consumers’ confidence. Food and feed that are free from dangerous substances are essential to our quality of life. Monitoring of the level of achievement goes hand in hand with the availability of faster and easier detection methods.
for such contaminants. The innovative methods that have been developed by the CONffIDENCE consortium allow both laboratory and on-site monitoring and improved chain management, thus keeping our food safe while preventing unnecessary waste of resources through spoilage or needless destruction. Through the application of these fast and inexpensive tests, European food and feed companies will be able to enhance their quality control and HACCP plans, thus safeguarding the food safety for European consumers and improving competitiveness of the European agro-food sector.

Several international surveys have been conducted within CONffIDENCE, viz. a combined survey of persistent organic pollutants (POPs), inorganic arsenic, methylmercury, perfluorinated compounds and polyunsaturated fatty acids (PUFA) in fish and surveys for paraquat and diquat in potatoes, cereals and pulses, for antibiotics in honey and for inorganic arsenic in rice. The datasets will be made available to EFSA and will contribute to the assessment of dietary intake of these contaminants. The combined survey on the co-occurrence of contaminants and PUFA in fish has yielded a unique European dataset that will contribute to the establishment of risk-benefit models.

The scientific achievements of CONffIDENCE were presented in many conferences related to food and feed safety, among others in two RAFA Conferences (2009, 2011), two International Feed Safety Conferences (2009, 2012), the EU-China Science and Technology Week / World Expo (2010), two MoniQA conferences (2010, 2013), Apimondia (2011), Aquaculture Europe (2011), the Food Integrity and Traceability Conference (2011), Euroresidue (2012) and WMF meets IUPAC (2012). Three very successful Open Days devoted to interaction with the main stakeholders were organized in conjunction with the Rapid Methods Europe Conference (2010), the International Triennial Meeting of the World Aquaculture Society (2011) and the 5th International Symposium on Recent Advances in Food Analysis (RAFA 2011). Moreover five stakeholder workshops have been organized, among others the final stakeholder workshop in Brussels in December 2012 for invited representatives from DG SANCO, DG RTD, EFSA, industrial food & feed branch organization, ETP-Food for Life, industrial foundations, COST-Actions, CEN-committees and relevant EC-supported projects. In total 9 biannual e-Newsletters have been issued, informing the registered stakeholders about the achievements of CONffIDENCE and including news from other scientific projects / events and news regarding relevant legislation. Through all these dissemination activities the scientific community, major stakeholders from European and national food and feed industries and governments, EFSA and CEN may utilize the major outcomes of CONffIDENCE for further improvements in food safety.

Until April 2013, 31 publications in peer reviewed journals have been published. Moreover, a special issue of Analytical and Bioanalytical Chemistry will be published in 2013 that will include approx. 20 papers on the main outputs of CONffIDENCE. Approx. 10 papers will be submitted to other peer reviewed journals. Through these publications, the scientific community will profit from the scientific advances achieved in CONffIDENCE.

For science education impact, CONffIDENCE organized an International Course on Advanced Food Analysis in 2010. This course was jointly organized with The Graduate School VLAG (Wageningen UR) and was attended by 60 PhD students and 6 Post-doc researchers. In 2011 a BSc education module on “Theoretical and technical issues on mycotoxins and plant toxins in food” was jointly organized with the CAH Dronten University of Applied Sciences (NL). This course was attended by 26 International students and 49 Dutch students. Through these courses international PhD and BSc students have improved their knowledge about rapid tests for food safety.
Regarding exploitation of results, some of the dipstick tests developed and validated within CONffIDENCE, viz. the 4-Mycosensor for mycotoxins in cereals (food and feed) and the Bee-4-sensor for antibiotics in honey, have already been commercialized by one of the partners, a Belgian SME. Moreover, a spin-off company will be created by one of the partners to commercialize antibodies and biosensor kits. Through CONffIDENCE, these SMEs will be able to strengthen their economic and IPR positions, show a steady growth and thus create more jobs in Europe. The Belgian SME partner (Unisensor) received the Trends Gazelles Ambassador 2012 award of the small-sized enterprises in the province of Liège for being the fastest growing enterprise in the last 5 years. Moreover, large-size European food and feed companies will be able to improve the safety of their products, thus improving competitiveness of the European agro-food sector.

**Address of the project public website / contact details**

<table>
<thead>
<tr>
<th>Project acronym</th>
<th>CONffIDENCE</th>
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<tbody>
<tr>
<td>Project full title</td>
<td>CONtaminants in Food and Feed: Inexpensive DETectioN for Control of Exposure</td>
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<tr>
<td>Duration</td>
<td>1 May 2008 – 31 December 2012</td>
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<tr>
<td>Coordinator</td>
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<td>E-mail</td>
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Project logo :