SAMPLE PRETREATMENT METHODS FOR THE DETERMINATION OF MYCOTOXINS AND OTHER HAZARDOUS SUBSTANCES (DIOXINS) IN FOOD AND FEEDING MATERIALS USING GAS CHROMATOGRAPHY AND DOUBLE FRAGMENTATION MASS SPECTROMETRY-MS/MS*

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Abstract

This paper describes newly invented methods which have been successfully used in the determination of mycotoxins, dioxins, PAHs, pesticide residues and other toxic organics in ppt level in the majority of sample matrices in our laboratory. A method used for Semipermeable Membrane (SPM) sample clean-up and preconcentration for the determination of toxic organic compounds in various matrices including industrial, environmental, biological and food and feedingstuffs is presented. This method is based on an isotope dilution procedure with the use of isotopically labelled internal and clean-up standards. Determination was performed using gas chromatograph equipped with double fragmentation mass spectrometric detector (GC-MS/MS system). Identification and confirmation was based on characteristic mass spectral data obtained from electron ionisation primary ions and helium atom collision secondary ions (MS/MS mode).

Key words: food, feedingstuff, SPM, clean-up, GC-MS/MS, analysis

Introduction

Mycotoxins are chemical substances that moulds create generally as secondary metabolites, thought to possibly play a role in either helping to prepare the substrate on which they exist for digestion, or as defence mechanisms, and some have suggested that they may be produced when the organisms are under stress, which could be related to competition or defence, or simply due to inhospitable environmental conditions. Some of the mycotoxins may also be neurotoxic (a toxin that is known to cause neurological damage), and most commonly reach people from the air, via spores from the moulds in question. They are also found in small particulates of mould that have dried and turned to dust. Spores, when inhaled, can colonize in the sinuses and throughout the body, including the brain, lung and gut after a period of time.

Dioxins have never been produced intentionally but can be found in many chemical products as trace contaminants; they may also form in combustion processes.

Three basic sources of dioxins have been identified and described: the chemical industry, smelters and sintering plants, and waste incinerators (Masuda et al. 1996; Liem and Theelen 1997; Rappe et al. 1991).

Within the chemical industry, the synthesis and processing of chemical compounds which contain chlorine atoms, the paper industry, and wastes from dry distillation processes, are the main sources of dioxins. Combustion processes include municipal, industrial and hospital waste, and sewage sludge incineration plants, enrichment of metal ores, thermal

processing of regenerated materials in recycling processes, and fuel combustion in power plants and car engines. Sewage sludge, composts and contaminated soil from areas close to dumps and chemical plants are processed wastes loaded with dioxins (Liem and Theelen 1997).

Liem and Theelen (1997) showed that the main route of dioxins exposure in human and animals is the consumption of contaminated food. Recent data show that background exposures are in the order of 100-200 pg-TEQ[♣] per human body per day e.g. 2-3 pg-TEQ/kg/day.

Sample extraction, enrichment and clean-up procedures have always played an important role in the determination of these toxic substances in food and feedingstuffs. The matrix complexity of most food samples mean that the sample preparation and pre-treatment may be a labour intensive and time consuming procedure (Rappe et al. 1991; Grochowalski and Wiatr 1998). The sample final extract must be free of most contaminants before it is introduced into the analytical instrument.

The general procedure as follows: sample extraction; sample clean-up, sample preconcentration and enrichment.

Depending on the concentration of natural lipids, triglicerides, unsaturated fatty acids and other organic matrices as well as organic compounds such as food flavours, colorants and stabilising agents introduced in industrial processes this sample pre-treatment may be achieved using many individual preparation techniques such as: Soxhlet Extraction (SE), Supercritical Fluid Extraction (SFE), Accelerated Solvent Extraction (ASE), Microwave Extraction (MWE), Ultrasonic Extraction (USE), Solid Phase Extraction (SPE) or the simplest but laborious and requiring a high volume of solvent Liquid/Liquid Extraction (LLE) for obtaining a sample of crude extract and extract clean-up.

Semi Permeable Membrane Dialysis (SPM)

However, the extraction process may be very time consuming. For example Soxhlet extraction can be as long as 18-24 hours for most of the samples. The development of fast and effective extraction has made it possible to reduce the time to minutes – for example Accelerated Solvent Extraction carried out at elevated temperature and pressure (Erickson 1997).

In most of extraction processes extract of heavy contaminated matrix is obtained.

[♣] TEQ is a value describing the relative toxicity of the sample analysed in relation to the total mass of the 17 most toxic dioxin congeners (PCDD/F) which contain chlorine atoms in positions 2,3,7 and 8 as well as for the three coplanar biphenyls (PCB#77, PCB#126, PCB#169). A detailed explanation of the TEQ value and methods for its calculation are given below.

The use of semipermeable membranes is a method of choice in this case. For lower molecular weight compounds like mycotoxins, dioxins, PAHs and other of relatively high toxicity permeability through the membrane is much higher than for matrix compounds. Therefore, very effective clean-up is available within 24 hours of dialysis through SPM membrane. Low amount of solvents, small laboratory area for single sample clean-up operation and no sophisticated instrumentation to be used make this method competitive to the other ones. Moreover, the recovery of analyte is on satisfactory level of 60 – 110% for most of mycotoxins, dioxins and other chlorinated and brominated aromatic organic compounds.

Procedures presented in this paper are only a schematic presentation of analytical operations and do not contain detailed information because of the individual sample matrix complexity.

Sample clean-up methods

Method 1. The first step in the sample clean-up – SPM dialysis

The appropriate mass of food or feedingstuff sample is freeze dried and extracted with dichloromethane to obtain minimum 5g of fat. A SPM membrane of 80 μm polyethylene foil tube (Exposmeter AB, Trehörningen 34, 922 66 Tavelsjö, Sweden) 2,5 cm wide, 20 cm length, with one end sealed, is pre-washed overnight with hexane. Then, the membrane is filed up with portion of 10 ml of solution based on 50% dichloromethane/hexane solvent containing 5g of animal or plant fat. Filled SPM tube is inserted in a large glass tube of 100 ml capacity and 80ml of hexane is poured into. The SPM is bent in the upper side and should be placed in hexane in that way, which allows to seal the glass tube with the glass stopper (fig. 4). Dialysis should be performed for the minimum of 24 hours but not longer than 48 hours. The glass tube is placed in light protected (Aluminium foil covered) large glass beaker. After the equilibrate of analyte concentration in and out of the membrane, the outer hexane solution is transferred to the measuring cylinder and the final volume is recorded (see figures 1 – 4).

Fig. 1. Single, new SPM diaphragm

Fig. 2. Diaphragm filled with sample of fat extract

Fig. 3. Introduction of 80 ml of hexane as a collective solvent

Fig. 4. Sample ready to dialysis for 24 hours

Clean-up of the crude extract using gel permeation chromatography

Solvent excess of the hexane extract from SPM is evaporated in a rotary evaporator up-to a few millilitres. Evaporation to dryness is not recommended because of severe analyte losses.

The concentrated hexane solution is introduced into the chromatographic column filled with 10g of freshly activated Silicagel of 60-200 mesh. The column is then washed out with

an appropriate solvent (e.g. for dioxins and zearalenone with hexane, for PAHs and chlorinated pesticides with dichloromethane) the solution is concentrated and transferred to the Bio-Beads SX-3 gel permeation chromatography column. The column is washed out with dichloromethane and the fraction, which contains the compound of interest is collected. The appropriate fraction should be adjusted before using the standard analyte solution. The appropriate fraction is concentrated in the rotary evaporator and finally under an inert gas stream up-to the volume of 20 – 50 µl. Nonane should be used as a keeper solution, by adding of 20-50µl portion just before the final solvent evaporation.

Method 2. Clean-up using the active carbon columns (for dioxins and PCB determination) – an alternative for SPM method

This method is based on the ability of dioxins and other planar, aromatic compounds to undergo strong adsorption to the surface of active carbon (Grochowalski and Wiatr 1998). This method is suitable for high fat content samples as food and feedingstuffs.

The food or feedingstuff sample after an extraction in dichloromethane in concentrated in the rotary evaporator and drying with anhydrous Na_2SO_4. The fat content is recorded by weighing the residue. After the fat dissolving in dichloromethane the solution is introduced into the glass column containing 0,5 of the specially pre-treated active carbon. After introduction of the sample, the column is washed out with small portions of dichloromethane, methanol and cold toluene.

The fraction, which contains dioxins as well as coplanar PCBs, is received when the column is turned upside down and thoroughly washed with hot toluene (at temperature close to its boiling temperature). The volume of toluene used for quantitatively dioxins desorption must be adjusted individually for each column. The blank sample containing ^{13}C-PCDDs, ^{13}C-PCDFs and ^{13}C-PCBs solution may be used for this purpose. The excess of toluene is then evaporated in a rotary evaporator, Kuderna-Danish apparatus or at least in an inert gas stream. When GC-MS/MS system is used for the analysis the sample can be concentrated up-to 50 µl without further purification. After introduction of 20 µl of syringe standard the solvent excess is evaporated and the final volume is adjusted with nonane.

Clean-up using Alumina columns.

In some cases for mycotoxins determination in heavily contaminated samples by chloroorganic pesticides, the extra clean-up step with 1g of basic Alumina placed in 5mm of inner diameter glass column, is required. After the sample introduction, the column is washed

with 2% dichloromethane in hexane, 50% dichloromethane in hexane and dichloromethane, respectively. The first fraction contains the most of mycotoxins (e.g. zearalenon) when the pesticides are to be eluted with subsequent fractions.

After completing the sample pre-treatment procedures the final extract is sealed in a labelled glass vial of appropriate volume (normally is 20 - 100 μl) and stored in refrigerator in temperature of 2-7^{0}C before the analysis. For compensation of possible, incidental loss of part of solvent during the long time of storage, the internal standard (IS) of recorded concentration is introduced before the extract is placed in the glass vial. It helps to control the IS concentration and the recalculation of the extract final volume.

Instrumental quantitative analysis

Gas chromatographic separation and mass selective detection (GC-MS) should be used for quantitative analysis of mycotoxins, dioxins and other trace organics. This method is useful for the determination of traces of analyte in the presence of other organic interferences in the final extract (Korfmacher et al. 1984, Rappe et al. 1991 and Liem and Theelen 1997). For this purpose our Laboratory uses ThermoQuest GCQplus GC-MS/MS system. The system consists of the Trace 2000CE gas chromatograph equipped with split/splitless injector, coupled with ion trap mass selective detector working in MS/MS mode.

Two different columns were used for chromatographic separation of PCDD/F congeners: DB-5MS (60m, 0,25 mm), low polarity capillary column for general use and DB-17 (30m., 0,25 mm) intermediate polarity column which performance is suitable for separation of 2,3,7,8-TCDF and 2,3,7,8-TCDD from the other tetrachlorodibenzofurans (TCDFs) and tetrachlorodibenzodioxins (TCDDs). The samples were injected using autosampler coupled to split/splitlesss injector.

Limit of detection for this method (LOD) is better than 0,1 pg in injection for 2,3,7,8-tetrachlorodibenzodioxin (TCDD) in animal fat samples. For mycotoxins LOD is better than 1 pg in injection for most of food and foodstuff samples.

The systems which can provide double fragmentation of the molecule of analysed compound (GC-MS/MS) can be considered as a compromise between investment and operating costs and demands to reach high selectivity of separation of analytical systems. GC-MS/MS system can be regarded as a newer version of quadruple detector or ion trap upgraded for detection of the so-called secondary ions formed in collision with the atoms of helium. The performance of the systems can be compared to the high-resolution, magnetic GC-MS systems if the secondary ions are formed in the optimised conditions. In recent years, the new

ideas appeared for mass detectors – GC-MS/MS systems. The basics of their work are similar to that of the ion trap. PCDDs and PCDFs molecules after leaving the chromatography column are directed to the ion source where they undergo ionisation with the thermal electrons usually with energy of 70 eV. In these conditions M^+ molecular ion for a PCDD or a PCDF molecule is formed. Moreover, fragmentation ions are formed but the number of them is much smaller. Molecular and fragmentation ions, which were formed, are directed through the so-called ion optic into a ring-shaped electrode (ion trap). Altering potential of this electrode enables to keep the ions in the area around the electrode and further, selective release to dynode of the photoelectric multiplier. However, it is possible to introduce a small amount of helium into it through a special valve.

As a result, molecular (primary) PCDD/F ions are accelerated in the ion optic system and undergo the collision with the atoms of helium. It results the formation of so-called secondary or daughter ions. The secondary ions are formed by splitting out of COCl particle from the molecular ion M^+. In this case the $M\text{-}COCl^+$ ion is produced. Computer programmes enable detecting either the molecular ions or the secondary ions. This is possible because the signals for the secondary ions are detected only when the molecular ions are present. This is the "sine qua non" condition. Therefore, even if molecules of contaminants other than PCDDs or PCDFs molecules undergo fragmentation and form ions of the same mass (or m/e value), it is very unlikely that they can form the secondary ions of the same mass obtained from the collision fragmentation of PCDD or PCDF molecular ion. This is the reason why the selectivity of this method is very high, which may be compared to high-resolution mass spectrometers. Application of MS/MS method makes possible to obtain high selectivity of analysis and very high level of detection of the analyte as 0,1 pg of 2,3,7,8-TCDD for a single injection of animal fat extract (Grochowalski and Chrząszcz 2000; Grochowalski and Wiatr 1998).

The ratio of intensity of ion current for the molecular and the secondary ions is very important factor for the identification of the compound under analysis in MS/MS system. In this case collision energy of the molecular ion with the atom of helium is optimised as well as the helium concentration in the ion optic (by a proper adjustment of helium valve). These are time-consuming operations and they need a multiple analysis of the same sample.

The collision energy of a molecule with the atom of helium is individually selected for each dioxin congener, respectively (Grochowalski and Chrząszcz 2000; Grochowalski and Wiatr 1998).

Therefore, the determination of these compounds should be carried out separately, however, in most applications it is possible in one analytical run. The modern mass spectrometric software, which is available with new MS/MS systems, allows for the simultaneous monitoring of even 20 individual ion masses (m/z values). It significantly simplifies the clean-up procedure for most of food samples, making the use of SPM technique the competitive alternative.

Result calculation

The calculation of dioxins and mycotoxins concentration in the samples is based on the determination of the peak areas of identified congeners referred to the standard natural substances and for recovery control ^{13}C-PCDDs/Fs and/or (if available) ^{13}C-labelled mycotoxin individual compounds.

Recovery of the analyte is calculated on the basis of the ratio of the peak areas for substances labelled with ^{13}C and ^{37}Cl (only for dioxins), which were introduced into samples prior analyses, during the sample preconcentration and evaporation. Those substances were also used for calibration of gas chromatographic equipment, the determination of column efficiency and linearity of the detector response.

The recovery value R_i of ^{13}C internal standards from the concentrated, final sample extract is calculated from the Equation 1:

$$R_i = \frac{H_i x C_i x V_E}{H_{i-AV} x m_i} \qquad (1)$$

H_i - peak height (or area) of ^{13}C internal standard in the sample

C_i - mass concentration of ^{13}C internal standard in the external calibration mixture (ng ml^{-1})

V_E - final extract volume of the sample (μl)

H_{i-AV} - average peak height (or area) of ^{13}C internal standard in the external calibration mixture

m_i - mass of ^{13}C internal standard introduced to the sample (pg)

Analyte mass concentration in the fat of the sample m_{ci} [ng kg^{-1} or pg g^{-1}] of analyte in the sample is calculated from Equation 2:

$$m_{ci} = \frac{H_A x C_{ST} x V_A x V_{C-inj}}{H_{ST} x m_S x R_i x V_{S-inj}} \quad [\text{ng kg}^{-1}\text{of fat}] \qquad (2)$$

H_A - peak height (or area) of the analyte in final sample solution

H_{ST} - peak height (or area) of the native compound in the calibration solution

C_{ST} - mass concentration of the native compound in the calibration solution [ng ml^{-1}]

V_A - final volume of sample solution [µl]

$V_{C\text{-}inj}$ - volume of injection of the calibration solution [µl]

$V_{S\text{-}inj}$ - volume of injection of final sample solution [µl]

m_s - sample fat mass [g]

R_i - ^{13}C- internal standard recovery value (calculated from Equation 1)

Dioxins TEQ calculation

The calculation of the toxic equivalency of the sample (TEQ) is based on the so-called toxic equivalent factors –TEF [10,11]. TEQ is the value, which is calculated from the result of analyses for mass concentration of all 17 congeners of PCDDs and PCDFs of which 2,3,7,8 positions in the molecule are substituted by the chlorine atoms. Numerical value of TEQ is the total value of partial parameters obtained by multiplication of the PCDD and PCDF congener mass the by the respective TEF value (Equation 3). TEF values are given in Table 1.

$$TEQ = \sum_{i=17}^{i=1} (m_i \times TEF_i) \qquad (3)$$

where:

TEQ – Summarised PCDD/PCDF Toxic Equivalency in pg

m_i – Mass of individual i-congener in pg.

TEF_i – Toxic equivalent factor for i-congener of PCDD/F , in relation to the toxicity of 2,3,7,8-TCDD

The numerical values for TEF are given in Table 1. They show a relative toxicity of each congener of PCDDs and PCDFs in relation to the most toxic 2,3,7,8-TCDD for which, according to WHO directives from 1998, this factor is 1. For the least toxic OCDD and OCDF, TEF at 0.0001 were accepted.

On the base of summarised mass concentration as ng-TEQ/kg of the sample, it is possible to determine a potential toxicity of sample analysed in relation to PCDDs, PCDFs.

Table 1. Values of TEF for PCDDs and PCDFs individual congeners (for TEQ calculation) accordingly to determination of dioxins in food and feedingstuffs (Council Regulation 2001).

PCDD congener	TEF	PCDF congener	TEF
2,3,7,8-TCDD	1	2,3,7,8-TCDF	0,1
1,2,3,7,8-P_5CDD	1	2,3,4,7,8- P_5CDF	0,5
1,2,3,4,7,8-H_6CDD	0,1	1,2,3,7,8- P_5CDF	0,05
1,2,3,6,7,8- H_6CDD	0,1	1,2,3,4,7,8- H_6CDF	0,1
1,2,3,7,8,9- H_6CDD	0,1	1,2,3,6,7,8- H_6CDF	0,1
1,2,3,4,6,7,8- H_7CDD	0,01	1,2,3,7,8,9- H_6CDF	0,1
OCDD	0,0001	2,3,4,6,7,8- H_6CDF	0,1
		1,2,3,4,6,7,8- H_7CDF	0,01
		1,2,3,4,7,8,9- H_7CDF	0,01
		OCDF	0,0001

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