DETERMINATION OF PCDD/Fs IN FRESH AND AGED DAIRY PRODUCTS BY GEL PERMEATION CHROMATOGRAPHY

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Introduction

The objective of this paper is to develop an analytical method for determining the concentration of PCDD/Fs in milk and its derivative products by gel permeation chromatography (GPC) coupling US EPA 1613[1] and 3640[2] methods. After extraction of the matrix, the organic extract is preliminarily purified on a stratified column composed, from the bottom upwards, of the following solid phases: anhydrous sodium sulfate, silica gel, 96% sulfuric acid mixed with Extrelut in a 2/1 (w/w) ratio, silica gel and anhydrous sodium sulfate; a second purification step is performed by means of GPC followed by Solid Phase Extraction (SPE) on alumina cartridges. Cleaned extracts are analyzed by HRGC/HRMS.

Materials and method

The analytical method is based on the extraction of the fat portion using solvent purification through acid column, gel permeation chromatography, alumina column cleanup followed by instrumental analysis by HRGC/HRMS. All the steps of the present method have been developed using ISO17034 certified $^{13}C_{12}$-labeled PCDD/Fs standards (Wellington Laboratories, Canada). The clean-up system used (PrepLinc LVi™ J2 Scientific, Missouri, USA) allows the use of several modules in series to constitute a completely automated system. Extraction of the portion of fat needed to reach the set LOQs is the first step. Only for fresh products as milk[3] and cream a liquid-liquid extraction has been carried out in a separatory funnel: $^{13}C_{12}$-PCDD/Fs extraction standards are added to an adequate sample quantity together with 1 g of sodium oxalate and stirred vigorously. Then 100 ml of methanol, 100 ml of diethyl ether and 100 ml of n-hexane are added; the separatory funnel is shaken vigorously and vented after the addition of each solvent. After about an hour of rest, a well-defined phase separation is obtained; the lower phase is discharged. The organic solvent is recovered by filtering through anhydrous sodium sulfate. Organic phase fat content is subsequently determined gravimetrically by evaporating the solvent to dryness (TurboVap™, Biotage, Sweden). The extraction technique for others fresh dairy products, such as mozzarella cheese and burrata cheese, consists of freeze-drying, followed by extraction using an accelerated solvent extractor (ASE - Thermo Fisher Scientific, USA) or Soxhlet. Aged dairy products, such as Grana Padano cheese and Parmigiano Reggiano cheese, are directly extracted by either Soxhlet or ASE. Table 1 shows some examples of the percentage content of fat for milk and other milk-derived products.

Table 1 - Percentage content of fat for milk and some dairy products

Dairy Product	Sample n.	% of fat	Fresh (F) or Aged (A)
Milk	30	3 – 8	F
Mozzarella cheese	6	15 – 25	F
Grana Padano and Parmigiano Reggiano cheese	67	20 – 35	A
Cream	26	20 – 40	F
Mascarpone cheese	7	30 – 50	F

Extracts were purified as follows:

1) Cleanup by acid column: a glass column (internal diameter 2 cm) equipped with a glass septum is prepared from the bottom upwards as follows: 2 cm Na_2SO_4 anhydrous; 3 cm silica gel, a mix of Extrelut (10 g) and H_2SO_4 at 96% (20 g); 3 cm silica gel; 2 cm Na_2SO_4 anhydrous. Extracted fat is re-dissolved n-hexane, then put on top of the column and eluted with 140 ml of n-hexane. The column sulfuric acid/Extrelut active phase is sufficient for cleaning up to 4 g of fat, for higher concentrations of fat the active phase can be increased. The cleaned extract is concentrated on a Turbovap, solvent exchanged to 5mL dichloromethane (DCM) for GPC cleanup and transferred to a washed test tube;

2) Fully automated clean-up system: vials coming from 1) are placed on the rack of a previously configured autosampler (ASL); and a working sequence, software generated and controlled, is able to join the purification steps reported below;
3) GPC Cleanup: 5 ml of the organic extract in DCM were quantitatively and automatically injected into a traditional GPC glass column (70g S-X3 Bio-Beads, J2 Scientific Missouri, USA); the output fraction containing PCDD/Fs is collected from 29 minutes up to 41 minutes, at the flow rate of 5 ml/min;
4) Evaporation: the collect fraction reported in 3) is fed to an evaporation device (AccuVap™) to perform a concentration and subsequent solvent exchange to n-hexane, at a final volume of about 2 ml which is quantitatively transferred to the following step.
5) SPE: the fraction reported in 4) is fed directly to alumina cartridges (5.3 g basic-alumina; J2 Scientific, Missouri, USA), pre-conditioned with n-hexane and eluted with two separate liquid phases: the first elution with a solution of n-hexane/dichloromethane 94/6 v/v (pre-eluate) which is sent to waste and the second elution with a solution of n-hexane/dichloromethane 50/50 (eluate) where PCDD/Fs are collected.

After preliminary solvent evaporation by TurboVap to 0.5 ml, the sample is transferred (n-hexane) into 0.25 ml inert glass insert and completely evaporated by N_2; finally the $^{13}C_{12}$-labelled syringe standard is added to the sample (the final volume is 20 µl) and it is analyzed by HRGC/HRMS (DFS - Thermo Fisher Scientific, USA). The process flow diagram is shown in figure 1.

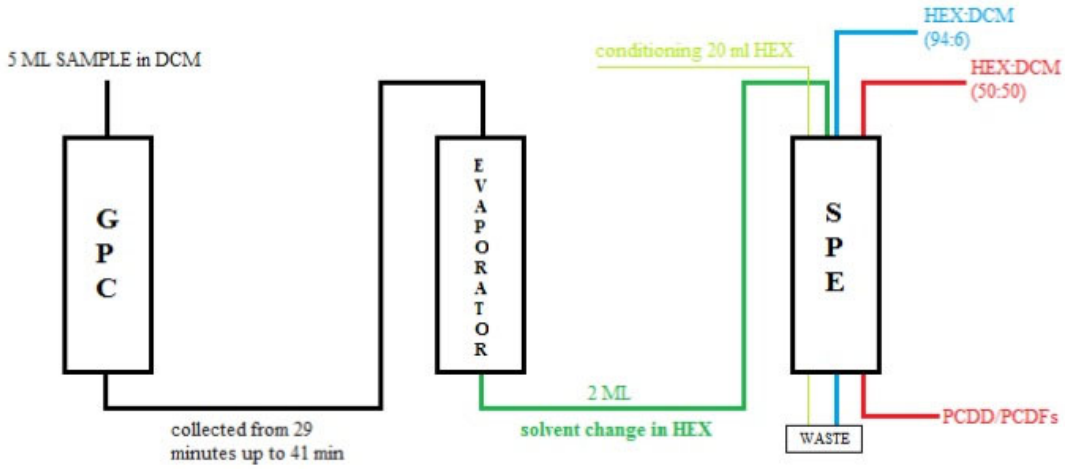

Figure 1 Fresh and aged dairy products automatic clean-up: process flow diagram

Result and discussion

Recovery percentages of $^{13}C_{12}$-labeled congeners in relation to those contained in the $^{13}C_{12}$-labelled syringe standards are of great importance concerning 2017/644 EU Regulation[4]. In order to ensure the quality of the data it requires that they are within the range between 60% and 120%. Figure 2 shows the values of the average percent recoveries of $^{13}C_{12}$-PCDD/Fs congeners in the 136 analyzed samples. They are all within the acceptance limit of EU regulations. The summary of the analyzed samples, mostly coming from the large-scale retail trade, represents native? values between 0.32 and 0.40 pg WHO-TEQ/g of fat (Figure 3), in conformity to the maximum levels indicated by 1259/2011 EU Regulation[5] which imposes the maximum threshold of 2.5 pg WHO-TEQ/g of fat. Based on the LOQs chosen by the laboratory, the value of 0.32 is the minimum value calculated in upper bound.

The automated analytical method shows good mean recoveries percentages for $^{13}C_{12}$-PCDD/Fs congeners in all analyzed samples as shown in Figure 1 and proves its robustness. In no case were the limits established by European regulations exceeded. The selectivity of the method allowed for control of any matrix effect and accuracy of identification chromatographic peaks. By resizing the acid column, it is also possible to adapt the present method to varying amounts of fat, being able to purify the extracts in a way that is compatible with the HRGC/HRMS instrumental analysis. The different dairy products already reported in Table 1 have also been sub-divided into two large categories: fresh dairy products and aged dairy products; then a statistical analysis of the average recoveries of the labeled? extraction standards has been performed.

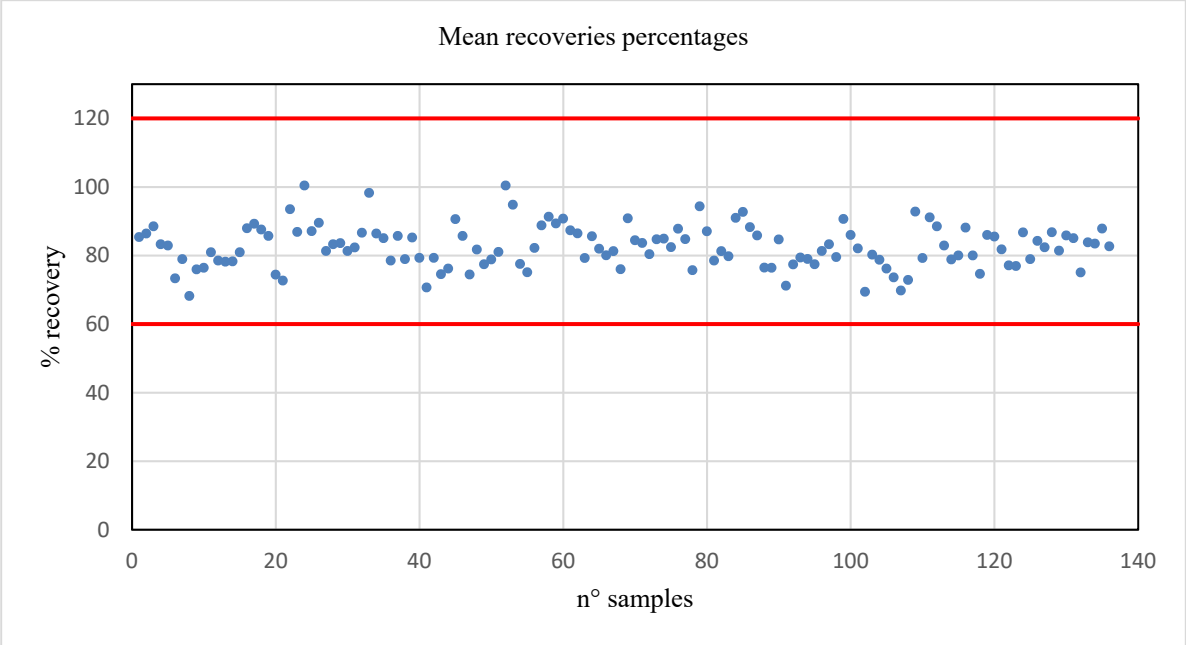

Figure 2 – Mean recovery percentages of $^{13}C_{12}$ PCDD/Fs congeners

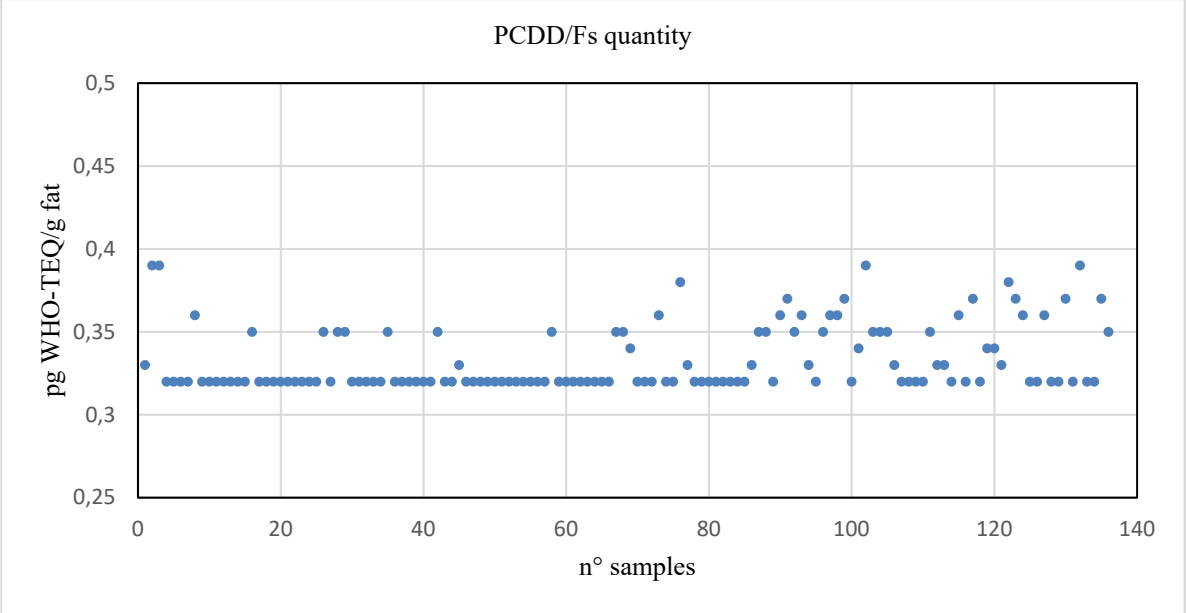

Figure 3 – PCDD/Fs equivalent toxicity in pg WHO-TEQ/g fat (WHO TEF 2005)

Specifically, a T-test for difference of means comparison has been applied in order to confirm if the recovery calculated on the two considered groups provide similar analytical results[6]. The outcome of this test is the acceptance or rejection of the null hypothesis (H_0), within a predefined confidence level generally at 95%. The null hypothesis states that any differences or outlying results are purely due to random and not systematic errors. The alternative hypothesis (H_1) states exactly the opposite. An erroneous rejection of H_0 (even though it is true) constitutes a "type 1 error" or *p-value*. A smaller p-value means that there is stronger evidence in favor of the alternative hypothesis. The most commonly used p-value is 0.05.

T-test for the comparison of two means assumes:

(a) A normal distribution for the populations of the random errors;

(b) There is no significant difference between the standard deviations of both population samples.

t_{exp} (experimental t value) is calculated from the two means $\bar{x}_A$ and $\bar{x}_B$, from the number n_A and n_B of data set A and data set B, respectively, and s_{AB} being the pooled estimate of standard deviation, according to:

$$t_{exp} = \frac{|\bar{x}_A - \bar{x}_B|}{s_{AB}\sqrt{\frac{1}{n_A}+\frac{1}{n_B}}}$$

To accept or reject H_0, t_{exp} value is compared with the theoretical t_{th} value corresponding to the given degree of freedom (df) N ($N = n_A+n_B-2$) and the confidence level chosen. If $t_{exp}>t_{th}$ then H_0 is rejected. In alternative, H_0 is rejected if $p<0,05$. Average recoveries calculated on the two groups of matrices, fresh (F) and aged (A), show that the difference between them is completely random and it does not depend on the maturing process as reported in Table 2.

Table 2 – Comparison between overall average recovery of fresh (F) and aged (A) dairy products

Matrix	F			A			t_{exp}	df	p-value
	n_F	Mean	Dev.st	n_A	Mean	Dev.st			
rec% TEQ	66	82,05	6,00	70	83,18	6,24	1,07	134	0,29

However, average recoveries calculated on PCDD/Fs congeners show different values between fresh and aged products. These differences for many congeners (highlighted in green in Table 3) have a statistical significance and they cannot derive from casualness. More research will be performed in the future to try to understand the

Table 3 – Comparison between average recoveries for PCDD/Fs congeners of fresh (F) and aged (A) dairy products

Matrix	F			A			t_{exp}	df	p-value
PCDD/Fs congeners	n_F	Mean	Dev.st	n_A	Mean	Dev.st			
2378-TCDD 13C12	66	75,0	7,8	70	76,0	8,9	0,695	134	0,488
12378-PeCDD 13C12	66	96,6	12,4	70	91,4	16,0	2,110	134	0,037
123478-HxCDD 13C12	66	79,9	7,7	70	81,2	6,8	1,045	134	0,298
123678-HxCDD 13C12	66	80,3	8,8	70	81,5	8,4	0,814	134	0,417
1234678-HpCDD 13C12	66	90,2	9,7	70	91,0	9,3	0,491	134	0,624
OCDD 13C12	66	83,2	11,5	70	86,7	10,9	1,822	134	0,071
PCDD tot		84,2	2,0		84,6	3,2	0,868	134	0,387
2378-TCDF 13C12	66	69,0	5,9	70	71,9	6,3	2,767	134	0,006
12378-PeCDF 13C12	66	87,9	8,7	70	85,1	8,9	1,854	134	0,066
23478-PeCDF 13C12	66	88,3	8,4	70	84,4	8,5	2,690	134	0,008
123478-HxCDF 13C12	66	75,5	7,4	70	78,5	7,7	2,690	134	0,022
123678-HxCDF 13C12	66	75,4	7,5	70	78,9	8,3	2,314	134	0,011
234678-HxCDF 13C12	66	77,8	7,6	70	81,8	9,0	2,575	134	0,006
123789-HxCDF 13C12	66	80,6	7,9	70	85,5	9,3	2,792	134	0,001
1234678-HpCDF 13C12	66	83,2	9,6	70	83,9	10,0	3,302	134	0,678
1234789-HpCDF 13C12	66	85,9	9,8	70	89,9	9,7	0,416	134	0,018
PCDF tot		80,4	1,2		82,2	1,1	9,126	134	0,000

possible causes of these chemical-physical effects considering that the labelled extraction standard is added to the fat phase immediately before the extraction. The only appreciable difference in the analytical preparation between the two groups of matrices is the freeze-drying of fresh dairy products that it is not performed in the aged ones.

Acknowledgements
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References

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