SEARCHING FOR ANTHROPOGENIC CONTAMINANTS IN HUMAN BREAST ADIPOSE TISSUES USING GAS CHROMATOGRAPHY-TIME-OF-FLIGHT MASS SPECTROMETRY.

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ABSTRACT

The potential of GC-TOF MS for screening anthropogenic organic contaminants in human breast adipose tissues has been investigated. Initially a target screening was performed for a list of 125 compounds which included persistent halogen pollutants (OC pesticides, PCBs, PBDEs), PAHs, alkylphenols, and a notable number of pesticides from the different fungicide, herbicide and insecticide families. Searching for target pollutants was done by evaluating the presence of up to five representative ions for every analyte, all measured at accurate mass (20 mDa mass window). The experimental ion abundance ratios were then compared to those of reference standards for confirmation. Sample treatment consisted of an extraction with hexane and subsequent normal-phase HPLC or SPE clean-up. The fat-free LC fractions were then investigated by GC-TOF MS.

Full-spectral acquisition and accurate mass data generated by GC-TOF MS also allowed the investigation of non-target compounds using appropriate processing software to manage MS data. Identification was initially based on library fit using commercial nominal mass libraries. This was followed by comparing the experimental accurate masses of the most relevant ions with the theoretical exact masses with calculations made using the elemental composition calculator included in the software.

The application of both target and non-target approaches to around 40 real samples allowed the detection and confirmation of several target pollutants including *p,p'*- DDE, HCB, and some PCBs and PAHs. Several non-target compounds that could be considered anthropogenic pollutants were also detected. These included 3,5-di-tert-butyl-4-hydroxy-toluene (BHT) and its metabolite 3,5-di-tert-butyl-4-hydroxybenzaldehyde (BHT-CHO), dibenzylamine, n-butyl benzenesulfonamide, some naphthalene-related compounds and several PCBs isomers not

included in the target list. As some of the compounds detected are xenoestrogens the methodology developed in this paper could be useful in human breast cancer research.

INTRODUCTION

Human exposure to environmental contaminants has been widely reported in the literature in the last few decades. Many of these contaminants, e.g. those known as persistent organic pollutants (POPs), are lypophilic in nature and their presence in the environment and fatty food is well documented. Contaminants like organochlorine (OC) pesticides, polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), polyaromatic hydrocarbons (PAHs) and alkylphenols, are frequently present in the environment and tend to bioaccumulate through the food chain. Consequently, these pollutants are commonly detected in human samples and, in some cases, their estrogenic potency has been reported [1, 2, 3, 4]. As a result, human exposure to these pollutants is a public concern for the general population and occupationally exposed people.

Robust and advanced methodology becomes necessary to investigate and confirm the presence of these pollutants in biological samples. A wide number of analytical methodologies and instrumentations are available nowadays, in most cases making use of gas chromatography (GC) combined with mass spectrometry (MS). GC-MS, operating in selected ion monitoring (SIM) mode, has been the most widely used in the determination of organic micro pollutants in environmental, food and biological samples. However, the analysis of complex matrices is still problematic due to interferences from matrix components, and the low selectivity of single quadrupole may not be sufficient for a reliable quantification and confirmation of the analyte. The special features of tandem mass spectrometry (MS/MS), using ion trap detectors (ITD) or triple quadrupole (QqQ) analyzers, has allowed the reduction and even the elimination of many of these interferences, with a notable improvement of selectivity. Tandem MS also offers better sensitivity and lower limits of detection by means of achieving appropriate precursor and product ion selection, thanks to the lower chemical noise in the chromatograms and much better signal-to-noise ratios. In recent years both

analyzers (ITD and QqQ) have been applied to multiresidue determination of organic pollutants in biological samples [5, 6, 7, 8, 9].

An inherent limitation of MS/MS techniques is their inability to detect untargeted compounds for which no data is acquired unless additional analysis is performed. Thus, it is not feasible to perform a search of compounds in a "post-target" way, i.e. investigating the presence of analytes (different to those pre-targeted) after MS data acquisition [10, 11]. Contrary to this, full spectrum techniques offer the advantage of performing retrospective analysis. That is a careful examination of old raw data sets looking for ions of other residues without the need to re-analyze the samples, provided a residue has passed the sample preparation, chromatographic separations and ionisation process with sufficient efficiency [12]. High-resolution time of flight mass spectrometry (TOF MS) is becoming an attractive and alternative full-spectrum technique for this purpose against traditional instruments such as double-focusing magnetic sectors. This technique which is highly expensive and needs highly skilled operators, has been employed for many years and is still being used to identify non-target compounds in samples using ion composition elucidation [13,14]. In addition, the investigation of non-target compounds using TOF MS is also possible using appropriate processing software making it feasible to manage the huge amount of data generated for samples. The unrivalled full spectrum sensitivity of this technique, together with its elevated mass resolution and excellent mass accuracy [15] make TOF MS very attractive for the rapid screening of target and non-target compounds and for their reliable accurate mass confirmation.

Our own research group has recently shown that GC-TOF MS allows a rapid and automatic accurate mass screening of target analytes using eXtracted Ion Chromatograms with narrow mass window (micro-window-XICs (mw-XICs) or narrow-window-XICs (nw-XICs)) to remove the chemical background and highly improve selectivity in the analysis of complex

matrices [16, 17]. For non-target analysis, the possibility of discovering the presence of compounds that had not been included in the initial target list is undoubtedly an attractive and challenging application that becomes more and more necessary as the number and type of organic contaminants in the environment continually increases. The use of component detection algorithms (CODA) and deconvolution software is required to both identify the presence of multiple components, and to deconvolute mass spectra for each individual component to be subsequently searched for in a commercial or home-made library. Accurate mass measurements notably facilitate the elemental composition calculation for every component which reduces the list of candidates, and makes the elucidation process of non-target compounds friendlier and helps solve ambiguous results in the library search.

Until now very few papers have been published dealing with trace analysis by GC-high-resolution TOF MS, and all have appeared within the last decade, which provides evidence of the novelty of this subject. GC-TOF MS applications have been described for the determination of PBDEs, pesticides, PAHs and PCBs in different environmental matrices [16, 17, 18, 19]. Other works deal with the determination of PBDEs, xenoestrogens, or flavour research in biological samples [5, 20, 21], or pesticides in food [22]. Recently, Čajka and Hajšlova reviewed the application of GC-TOF MS in food analysis [23].

The aim of this work is to investigate the capabilities of GC-TOF MS for screening a list of 125 target organic pollutants in human adipose breast tissues. Around 30 persistent halogen pollutants, for which the extraction and cleanup procedure had been optimized in a previous work [5], have been investigated as “pre-target” analytes and the results have been compared to previous data obtained by GC-(QqQ)MS/MS. Then, making use of the full spectral acquisition data acquired, and without re-analyzing the samples, around 100 more contaminants, including other PCBs, PBDEs, PAHs, alkylphenols, and a notable number of pesticides, like insecticides (organophosphates, carbamates and pyretroids), herbicides

(triazines and chloroacetanilides) and fungicides, have been selected to perform a "post-target" screening of real-world samples. Finally, searching unknown compounds present in breast tissue (non-target analysis) was carried out using CODA, deconvolution potential and the valuable accurate mass information provided by TOF MS.

EXPERIMENTAL

Reagents

In relation to the list of target analytes, reference standards of pesticides, octyl/nonyl phenols, PCBs (Mix 3, 100 µg/mL in cyclohexane) and PAHs (Mix 25, 100 µg/mL) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Acenaphthene and naphthalene (Fluka, Buchs, Switzerland) and fluoranthene (Riedel de Haen, Seelze, Germany) were also used. Standards of PBDEs (50 µg/mL in nonane) were obtained from Wellington Laboratories (Guelph, Ontario, Canada). In the case of solid reference standards, stock solutions (around 500 µg/mL) were prepared by dissolving reference standards in acetone and stored in a freezer at –20°C. Working solutions for sample fortification and for injection in the chromatographic systems were prepared by diluting stock solutions in n-hexane.

Ethyl acetate, acetone and n-hexane were ultra-trace quality and purchased from Scharlab (Barcelona, Spain). Anhydrous sodium sulfate of pesticide residue quality (Baker, Deventer, Holland) was dried for 18 hours at 300°C before use.

Three isotopically labeled surrogates were used: hexachlorobenzene (HCB)-$^{13}C_6$ (Cambridge Isotope Labs, Inc. Andover, MA, USA), *p,p'*-DDE-D_8 and β-endosulfan-D_4 (Dr. Ehrenstorfer). Working solutions of labeled standards (~1µg/mL) were prepared by dilution of stock solution with hexane and stored at 4ºC.

Samples

Human breast tissues were obtained from women with breast cancer. Samples were collected from volunteer women at the Cancer Foundation's Oncology Institute in Valencia (FIVO). Adipose tissues were obtained from biopsies taken during breast surgery. For this study informed, written consents were obtained from the women beforehand. Samples were collected in sterilized polyethylene recipients, identified (devoid of personal identifiers) and immediately frozen. Two different samples were collected from each woman: adipose breast tissue and tumor fragment; with a total of 42 samples being analyzed, corresponding to 21 patients.

Instrumentation

The normal-phase (NP) HPLC system used for sample cleanup was based on our previous work [5]. For the GC instrumentation, an Agilent 6890N GC system (Paloalto, CA, USA) equipped with an Agilent 7683 autosampler was coupled to a time-of-flight mass spectrometer, GCT (Waters Corporation, Manchester, UK), operating in electron ionization (EI) mode. The GC separation was performed using a fused silica HP-5MS capillary column with a length of 30 m x 0.25 mm i.d. and a film thickness of 0.25 μm (J&W Scientific, Folson, CA, USA). The oven temperature was programmed as follows: 90ºC (1 min); 5ºC/min to 300ºC (2 min). Splitless injections of 1 μL sample were carried out. Helium was used as carrier gas at 1 mL/min.

The interface and source temperatures were both set to 250ºC and a solvent delay of 3 minutes was selected. TOF MS was operated at 1 spectrum/s acquiring the mass range *m/z* 50-650 and using a multi-channel plate voltage of 2700V. TOF-MS resolution was about 8500 (FWHM) at *m/z* 612. Heptacosa, used for the daily mass calibration as well as lock mass, was

injected via syringe in the reference reservoir at 30ºC for this purpose. The *m/z* ion monitored was 218.9856. The application manager TargetLynx, a module of MassLynx software, was used to process the qualitative and quantitative data obtained from standards and samples for target compounds. The application manager ChromaLynx, also a module of MassLynx software, was used to investigate the presence of non-target compounds in samples. Library searching was performed using the commercial NIST library.

Analytical procedure

Sample preparation and extraction

Between 0.1 and 0.5 g of tissue sample was spiked with 0.5 mL isotopically labeled surrogate solution (500 ng/mL), then homogenized with 5-10 g anhydrous sodium sulfate and extracted three times with 5 mL of n-hexane each time, shaking in vortex. After filtration, the extract was pre-concentrated under a gentle nitrogen stream at 40ºC, and the final residue was adjusted to 5 mL (normal-phase HPLC cleanup) or to 10 mL with hexane (SPE cleanup) depending on the subsequent cleanup applied.

Cleanup Procedure

Two cleanup methodologies were applied to the sample hexanic extracts, both based on our previous works [5, 8].

28 samples (14 samples of adipose breast tissue and 14 of tumoral fragment from 14 patients) were submitted to HPLC cleanup with a silica column, using two complementary procedures, and injecting 1 mL of hexanic extract into the LC system in each case [5]. The mobile phase was n-hexane (procedure A) or n-hexane/ethyl acetate (95:5 v/v) (procedure B), at a flow rate of 1 mL/min. In both procedures, after 16 min of injecting the sample extract, a pulse of 4 mL of ethyl acetate was introduced. Fractions eluting between minutes 1 and 17 (procedure A), and between minutes 4 and 17 (procedure B), were collected and preconcentrated under a

gentle nitrogen stream at 40ºC down to 1 mL. The two final cleaned-up extracts were injected separately into the GC-TOF MS instrument.

14 samples (7 samples of adipose breast tissue and 7 of tumoral fragment from 7 patients) were submitted to SPE cleanup [8]. 10 mL of the sample hexanic extract was passed through the silica SPE cartridge previously conditioned by passing through 6 mL of hexane. The first 3 mL was discarded and the rest, approximately 7 mL, was collected together with an additional fraction eluted with another 3 mL of hexane. The cleaned-up extract was preconcentrated to dryness under a gentle nitrogen stream at 40ºC and redissolved in 0.5 mL of hexane before GC-TOF MS analysis. This SPE procedure led to more concentrated extracts (0.2-1 g sample/mL) in comparison to the HPLC cleaned-up extracts (0.02-0.1 g sample/mL).

RESULTS AND DISCUSSION

In our previous work, a number of human breast tissue samples were extracted with hexane and purified by the two complementary NPLC cleanup procedures. Target analyses were performed by GC-(QqQ)MS/MS for around 30 organohalogen xenoestrogen compounds. The application of this methodology led to the detection and quantification of low levels of several analytes, mainly *p,p'*-DDE, HCB, β-HCH and some PCBs [5].

In the present work, we reanalyzed 28 of these samples by GC-TOF MS to confirm the presence of the analytes found by GC-(QqQ)MS/MS. Furthermore, taking advantage of the full spectrum acquisition in TOF MS, the presence of some other selected compounds was also investigated in a "post-target" way, without re-analyzing the samples [10, 24]. In addition, the elucidation of several unknown compounds (non-target analytes) was tested. The methodical approach previously developed for screening and confirmation of organic micropollutants in water [16, 17] was applied in this paper for searching target and non-target anthropogenic contaminants in human breast adipose tissues.

In addition another 14 sample extracts were cleaned-up by SPE and analyzed by GC-TOF MS applying both target and non-target approaches. Final extracts were more concentrated in these samples compared to those of HPLC clean-up, which facilitated the detection of non-target contaminants as will be shown in the next sections.

Target screening

GC-TOF MS confirmation of target analytes in samples was carried out by obtaining up to 5 micro window eXtracted Ion Chromatograms (mw-XIC), with a mass window of 0.02 Da, at selected m/z ions for every compound. The software application TargetLynx^{TM} was employed to automatically process data and to confirm the identity of target compounds detected in samples. Analyte confirmation was performed by comparing the experimental Q/q intensity ratios in samples with the theoretical ones, calculated from injection of standards in solvent. Q/q was the ratio between the most abundant ion (Q, quantitative) and every one of the other measured ions (q, confirmative). The presence of at least 2 ions measured at their accurate mass and the attainment of their Q/q intensity ratio within specified tolerances was required for the reliable confirmation of target analytes. Maximum deviations accepted were based on the European Commission Decision (2002/657/EC) [25], as it has been applied in previous works [5, 17, 26, 27, 28].

As stated in the introduction section, the sensitive full spectrum acquisition is feasible together with accurate mass measurements when working with TOF MS. This makes the application of two different approaches possible when facing target analysis [16, 17]: a) "pre-target" analysis, where the compounds are selected before analysis, reference standards are normally injected, and the methods are fully validated making quantification feasible in most of cases; b) "post-target" analysis, where the compounds are selected and searched after MS data acquisition. In this way, there is almost no limitation to the number of compounds that

can be investigated, but obviously they have to achieve the GC and MS analysis requirements. Typically, a "post-target" analysis is focused on identification and/or confirmation of compounds detected, not on quantification, and reference standards are not necessarily injected as the abundant and rich information provided by the instrument is sufficient for the identification of the compound.

In the present work, around 30 organic micropollutants (OC pesticides, PCBs and PBDEs), for which the extraction and cleanup procedures were previously validated using GC-(QqQ)MS/MS for measurements, were selected as "pre-target" analytes. Once the samples were analyzed, a notable number (around 100 compounds) of organic contaminants (other PCBs and PBDEs, PAHs, alkylphenols and other pesticides) were also searched for after MS acquisition ("post-target" analysis). Supporting information (**Table S-1** shows the list of target compounds selected in this work).

Regarding "pre-target" analysis, all reference standards were available because the sample procedure had previously been validated. Calibration standards were injected into the GC-TOF MS to evaluate the sensitivity of this technique and to estimate the lowest concentration for which the correct identification of the compound was experimentally feasible. This was done by measuring at least two ions with their Q/q ratio falling within specified tolerances [17]. In spite of the fact that TOF sensitivity in full acquisition is excellent, detection and confirmation of compounds was not feasible at concentrations as low as is possible using triple quadrupole working in SRM mode (see **Table 1**). As a consequence, the majority of the previous GC-(QqQ)MS/MS positive findings were confirmed by GC-TOF MS with the exception of those samples where analyte concentrations were below the detection capabilities of TOF MS. As **Table 1** shows, all positives of *p,p'*-DDE and 95% of the positives of HCB, β-HCH and PCB 153 were confirmed by TOF MS, as their concentration levels were high enough to be confirmed by this technique (>10 ng/g for *p,p'*-DDE, HCB and

PCB 153; and >50 ng/g for β-HCH). Contrary to this, the presence of oxychlordane, PCB 101, *p,p'*-DDD, *p,p'*-DDT and mirex could not be confirmed by TOF MS due to their low concentrations in samples (<10 ng/g for mirex and PCBs 101 and 153; <50 ng/g for oxychlordane and *p,p'*-DDD; and <250 ng/g for *p,p'*-DDT).

Among the remaining 66 positives (*trans*-nonachlor, PCBs 28, 118, 138 and 180), up to 41 were confirmed by TOF MS. In all cases where confirmation was not feasible, the reason was the lower sensitivity of this technique, as analyte concentrations were below 10 ng/g for PCBs 28, 118, 138 and 180 and below 50 ng/g for *trans*-nonachlor. As an illustrative example, **Figure 1** shows the extracted ion chromatograms (GC-TOF MS) and the SRM chromatograms (GC-(QqQ)MS/MS) for two positives of *p,p'*-DDE (445 ng/g) and HCB (428 ng/g) that were detected in an adipose tissue sample and could be confirmed by the two techniques. In both cases, the presence of chromatographic peaks at expected retention time and the attainment of all Q/q ratios when comparing to the reference standard allowed the confirmation of these findings in the samples. Additionally, the corresponding EI accurate mass spectra generated by TOF MS are shown. Mass errors for five representative ions were typically below 1.5 mDa, which gave more confidence to the confirmation process.

The complete spectral information acquired by GC-TOF MS allowed us to perform a "post-target" analysis. Thus, a strategy previously applied at our laboratory [17] was used for the determination of almost 100 compounds in the 42 samples processed. Among all the compounds investigated, four PAHs (naphthalene, phenanthrene, fluoranthene and pyrene) and six PCB congeners were detected in several samples (**Table 1**). **Figure 2** shows illustrative XICs for PCB 157, naphthalene and pyrene detected in adipose tissue samples. In addition to the accurate mass measurements, reliable confirmation was feasible as all Q/q ratios were within specified tolerances. Experimental EI accurate mass spectra generated by TOF MS led to mass errors for five representative ions always below 1.9 mDa. None of these

compounds were determined previously by GC-(QqQ)MS/MS, as their optimal SRM transitions had not been acquired when analyzing the samples.

Non-target screening

In this work, non-target screening in human breast tissue extracts was carried out by applying the ChromaLynx^{TM} Application Manager. This software automatically detected peaks with a response over user-defined parameters, displayed their deconvoluted mass spectra to be searched in the library, and produced a hit list with positive matches (library match >700 was used as criterium). The formulas from the library hit were submitted to the elemental composition calculator and the 5 most intense ions were scored by exact mass measurement for the confirmation/rejection of the finding [17].

All samples analyzed were processed using the described software. As shown in **Table 1**, several of the "pre-target" compounds were confirmed using this approach, in spite of the fact that no information or restriction was entered into the system, i.e. treating all compounds as actual unknowns. It is worth noting that the methodology employed was able to detect unknown components which were subsequently confirmed to be well known OC contaminants. Most of detections corresponded to HCB, *p,p´*-DDE and PCB 138, 153 and 180. The main limitation of this approach was its lower capability to discover the presence of components present at low concentrations in samples, only leading to satisfactory results in those cases where the components signal was significantly higher than background levels [17]. Obviously, this methodology is less powerful at low concentrations than target methods that have been purpose developed and validated, searching for the optimum analytical conditions for a limited number of target analytes. However, there are evident advantages for screening purposes because of much wider possibilities for detecting many other contaminants that would remain ignored in a target analysis.

The automated library search using extensive libraries (e.g. NIST) led to a large list of compounds identified using the non-target approach. Within this large list, only a few not included as target analytes were considered as potential contaminants (**Table 1**). Thus, 3,5-di-tert-butyl-4-hydroxy-toluene (BHT) was identified in 31 out of 42 samples. BHT is a synthetic, highly lipid-soluble antioxidant added in rubber, petroleum products and plastics, which is commonly used for preservation of food, cosmetics and other lipid-containing products. This antioxidant has exhibited contradictory actions on cancer growth as it has been shown to inhibit growth in some studies, and increase it in others. Therefore its toxicological implications are in permanent revision [29]. Several studies have already proven the presence of this compound in the aquatic environment [30, 31], food [32, 33, 34] and in adipose tissue [35]. Some of its metabolites, such as 3,5-di-tert-butyl-4-hydroxybenzaldehyde (BHT-CHO) and the dimer of BHT, 1,2-bis-(3,5-di-tert-butyl-4-hydroxyphenyl)ethane (2-BHT) seem to cause a human health risk. In our work, the metabolite BHT-CHO was also identified in one adipose tissue sample. **Figure 3** shows the positive finding of BHT-CHO in adipose tissue sample. In this case, two library spectra fitted with the experimental spectrum (forward match of 724 for BHT-CHO, and 705 for 2,6-bis(1,14-dimethylethyl)-4-ethyl-phenol). However, the accurate mass scoring, automatically performed by the software for four representative ions led to the confirmation of the identity of BHT-CHO with mass errors below 2 mD for three ions, and 3.2 m Da for the fourth. Mass errors were automatically calculated and they corresponded to the difference between the experimental accurate masses of the ions and the theoretical exact masses given by the elemental composition calculator.

N-butyl benzenesulfonamide (N-BBSA) used in polyamide and copolyamide plastics and in the manufacturing of sulfonyl carbamate herbicides, was found in one adipose tissue sample. Several authors have reported the presence of this compound, considered as neurotoxic to laboratory mammals, in the aquatic environment [36, 37, 38]. **Figure 4** shows the positive

finding of N-BBSA in an adipose tissue sample using the non-target approach. In this case, two library spectra fitted with the experimental one (forward match of 727 for N-BBSA, and 695 for bensulide), with low mass errors (≤ 2.4 mDa) for the three most abundant ions for both possible candidates, making the selection of the right structure troublesome. However, using a software option based on an isotope prediction filtering (i-FIT) of the sulfur atom, it was possible to discard the wrong structure. The filtering was carried out for two fragments with different number of sulfur atoms in the candidates (m/z_1 141.0033 and m/z_2 170.0300). As shown in **Figure 4**, both selected fragments fitted better with the presence of only one sulfur atom in the molecule (the lower the i-FIT value the better the fit). Consequently, N-BBSA was selected as the appropriate structure which was subsequently confirmed by the retention time match when injecting a reference standard in solvent.

Two naphthalene-related compounds (2-methylnaphthalene and 1,2-dimethylnaphthalene) were also identified in a few samples **(Table 1)**. The bioaccumulation of these PAHs in mussels has been investigated by other authors [39]. Additionally, positive findings of other PCB congeners, not included in the "pre-target" and "post-target" list, were detected in several samples. As no standards of these PCB congeners were available in the laboratory, the identification of each individual congener was not feasible using retention times, but the library fit and the accurate mass confirmation of up to five ions allowed confirmation of these congeners which contained 4, 5, 7 and 8 chlorine atoms.

In addition, 10 of the samples were submitted to a second extraction with 5 mL ethyl acetate after extraction with hexane. After preconcentration to 1 mL, the ethyl acetate extracts were directly injected into the GC-TOF MS for both target and non-target analysis, with the objective of investigating the presence in the samples of more polar compounds. N-(phenylmethyl)-benzenemethanamine, also called dibenzylamine, a thermal decomposition product of the vulcanization agent zinc dibenzyldithiocarbamate and a possible precursor to

the formation of N-nitrosodibenzylamine [40], was discovered in 4 out of 10 ethyl acetate extracts. Dibenzylamine has been also found by other authors in artificial saliva leachates from baby bottle teats [40] and in human-plasma and urine samples [41]. **Figure 5** shows a positive of dibenzylamine in an adipose tissue sample when using CODA and the deconvolution process. Accurate mass confirmation automatically performed by the software for four representative ions led to the confirmation of the identity of dibenzylamine with mass errors always below 3.4 mDa. For additional confirmation, a standard of this compound was acquired and injected in the system to check the retention time and spectrum and to unequivocally confirm the presence of this compound in the sample (see **Figure 5**).

CONCLUSIONS

GC-TOF MS has been proven to be a rapid and efficient technique for the screening and confirmation of anthropogenic contaminants in human breast adipose tissues. The evaluation of up to 5 micro window-extracted ion chromatograms (0.02 Da) at selected m/z ions and the attainment of their Q/q intensity ratios allowed the detection of several target compounds (HCB, β-HCH, *p,p'*-DDE, *trans*-nonachlor, and some PCBs). These were within a group of 30 selected analytes for which the extraction and cleanup procedures had been previously validated. Additionally, after MS data acquisition, the presence of around 100 additional compounds was investigated in a "post-target" way, without the need to reanalyze the samples. In this way, several PAHs (naphthalene, phenanthrene, fluoranthene, pyrene) and some PCB congeners (114, 123, 156, 157, 167 and 189) were found in several samples. In both cases, searching for the presence of the ions, measured at accurate mass, was performed in an automated and simple way using potent software. From the list of 30 "pre-target" analytes, a total number of 187 positives found in previous analyses performed in human breast tissues by GC-(QqQ)MS/MS with triple quadrupole were investigated by GC-TOF MS. 134 of these

positives were confirmed by GC-TOF MS, the difference being due to the lower sensitivity of this technique compared to GC-(QqQ)MS/MS in SRM mode, which hampered some detections at low analyte concentrations. However, the possibility of performing a "post-target" screening as a consequence of the full spectrum acquisition in TOF MS allowed the identification of other selected contaminants, like some PAHs and other PCB congeners, which had not been included in the initial list of target analytes and consequently could not be investigated by GC-MS/MS, in this way illustrating the potential of GC-TOF MS for screening purposes.

The application of a component detection algorithm (CODA) and subsequent deconvolution software has been found to be an attractive way to perform non-target screening. This has allowed the discovery of several compounds that were not included in any of the lists of target analytes, like BHT, BHT-CHO, dibenzylamine, N-BBSA, 1,2-dimethylnaphthalene, 2-methylnapththalene and other PCB congeners. The methodology applied in non-target analyses also allowed the detection and confirmation of a notable number of positives found in previous target analyses, i.e. the system was able to detect and confirm several compounds present in samples in spite of the fact that they were treated as unknowns. These findings were only feasible when analyte concentration was relatively high. Thus, the screening of anthropogenic contaminants in biological samples, where samples and analytes are treated as unknowns using component detection algorithm and deconvolution software, may not be completely satisfactory at the moment as the success of this approach gets notably worse at low concentrations. Both target analysis, focused on priority contaminants, and non-target analysis, is complementary and both are required to obtain the maximum sample composition information possible.

The use of non-specific libraries (e.g. NIST) leads to a large list of possible components in the samples, the majority of them irrelevant for the screening purposes. This fact makes the

selection of “relevant” compounds arduous for the analyst, as lists of many potential candidates have to be reviewed before the presence of anthropogenic contaminants can finally be reported. The availability of specific libraries purpose made for the type of research performed would make the work more user-friendly and would facilitate the discovery of contaminants in samples.

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FIGURE CAPTIONS

Figure 1. (A) GC-TOF MS extracted ion chromatograms at different m/z (mass window 0.02 Da) for "pre-target" *p,p'*-DDE and HCB detected in adipose breast tissue. (B) GC-(QqQ)MS/MS chromatograms for *p,p'*-DDE and HCB in the same sample as in (A). (C) Experimental EI accurate mass spectra.

Q, quantitative ion/transition; q, confirmative ion/transition; St, reference standard; S, sample; ✓, Q/q ratio within tolerance limits.

Figure 2. GC-TOF MS extracted ion chromatograms at different m/z (mass window 0.02 Da) for "post- target" PCB 157, naphthalene and pyrene detected in adipose breast tissue (top). Experimental EI accurate mass spectra (bottom).

Q, quantitative ion; q, confirmative ion; St, reference standard; S, sample; ✓, Q/q ratio within tolerance limits.

Figure 3. Identification of non-target 3,5-di-tert-butyl-4-hydroxybenzaldehyde (BHT-CHO) in an adipose tissue sample: (A) extracted-ion chromatograms for four BHT-CHO ions used for deconvolution. (B) Library mass spectrum of the candidate BHT-CHO at nominal mass. (C) Library mass spectrum of the candidate 2,6-bis(1,1-dimethylethyl)-4-ethyl-phenol at nominal mass. (D) Deconvoluted accurate mass spectrum of BHT-CHO in adipose tissue sample. (E) Library forward fit and accurate mass confirmation of 4 fragments; experimental accurate masses compared to theoretical exact masses (in brackets, mass errors in mDa) for the two possible candidates.

Figure 4. Identification of non-target N-butyl benzenesulfonamide (N-BBSA) in an adipose tissue sample: (A) extracted-ion chromatograms for four N-BBSA ions used for deconvolution; (B) Library mass spectrum N-BBSA at nominal masses; (C) Library mass spectrum of bensulide; (D) Deconvoluted accurate mass spectrum of N-BBSA from the adipose tissue sample; (E) Library forward fit and accurate mass confirmation of 4 fragments for experimental accurate masses compared to the theoretical exact masses (in brackets, mass errors in mDa) for two possible candidates.

Figure 5. Identification of non-target N-(phenylmethyl)-benzenemethanamine, (dibenzylamine) in an adipose tissue sample by GC-TOF MS: (A) extracted-ion chromatograms for four dibenzylamine ions used for deconvolution. (B) Commercial library mass spectrum of dibenzylamine at nominal mass. (C) Deconvoluted accurate mass spectrum of dibenzylamine from the adipose tissue sample. (D) Library forward fit and accurate mass confirmation of 4 fragments; experimental accurate masses compared to theoretical exact masses (in brackets, mass errors in mDa). (E) Extracted ion chromatogram at m/z 196.1126 for a solvent standard of dibenzylamine (100µg/L) and its corresponding experimental EI TOF spectrum.

REFERENCES

1 Ibarluzea JM, Fernández MF, Santa-Marina L, Olea-Serrano MF, Rivas AM, Aurrekoetxea JJ, Exposito J, Lorenzo M, Torne P, Villalobos M, Pedraza V, Sasco AJ, Olea N. Breast cancer risk and the combined effect of environmental estrogens. *Cancer Causes and Control* 2004; **15**: 591-600.

2 Cheng JP, Yuan T, Wu Q, Zhao WC, Xie HY, Ma YG, Ma J, Wang WH. PM10-bound polycyclic aromatic hydrocarbons (PAHs) and cancer risk estimation in the atmosphere surrounding an industrial area of Shanghai, China. *Water, Air and Soil Pollution* 2007; **183**: 437-446.

3 Brody JG, Moysich KB, Humblet O, Attfield KR, Beehler GP, Rudel RA. Environmental pollutants and breast cancer: Epidemiologic studies. *Cancer.* 2007; **109**: 2667-2711.

4 Meerts IATM, Letcher RJ, Hoving S, Marsh G, Bergman A, Lemmen JG, van der Burg B, Brouwer A. In vitro estrogenicity of polybrominated diphenyl ethers, hydroxylated PBDEs, and polybrominated bisphenol A compounds. *Environmental Health Perspectives* 2001; **109**: 399-407.

5 Hernández F, Portolés T, Pitarch E, López F. J, Beltrán J, Vázquez C. Potential of gas chromatography coupled to triple quadrupole mass spectrometry for quantification and confirmation of organohalogen xenoestrogen compounds in human breast tissues. *Analytical.Chemistry* 2005; **77**: 7662-7672.

6 Gómara B, Herrero L, Bordajandi LR, González MJ. Quantitative analysis of polybrominated diphenyl ethers in adipose tissue, human serum and foodstuff samples by gas

chromatography with ion trap tandem mass spectrometry and isotope dilution. *Rapid Communications in Mass Spectrometry* 2006; **20**: 69-74.

7 Barr DB, Needham LL. Analytical methods for biological monitoring of exposure to pesticides: A review. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences* 2002; **778**: 5-29.

8 Medina CM, Pitarch E, Lopez FJ, Vazquez C, Hernandez F. Determination of PBDEs in human breast adipose tissues by gas chromatography coupled with triple quadrupole mass spectrometry. *Analytical and Bioanalytical Chemistry* 2008, **390**: 1343-1354.

9 De Saeger S, Sergeant H, Piette M, Bruneel N, Van De Voorde W, Van Peteghem C. Monitoring of polychlorinated biphenyls in Belgian human adipose tissue samples. *Chemosphere.* 2005; **58**, 953-960.

10 Hernández F, Pozo OJ, Sancho JV, López FJ, Marín JM, Ibáñez M. Strategies for quantification and confirmation of multi-class polar pesticides and transformation products in water by LC-MS2 using triple quadrupole and hybrid quadrupole time-of-flight analyzers. *TrAC- Trends in Analytical Chemistry* 2005; **24**: 596-612.

11 Sancho JV, Pozo OJ, Ibáñez M, Hernandez F. Potential of liquid chromatography/time-of-flight mass spectrometry for the determination of pesticides and transformation products in water. *Analytical and Bioanalytical Chemistry* 2006; **386**: 987-997.

12 Nielen MWF, van Engelen MC, Zuiderent R, Ramaker R. Screening and confirmation criteria for hormone residue analysis using liquid chromatography accurate mass time-of-flight, Fourier transform ion cyclotron resonance and orbitrap mass spectrometry techniques. *Analytica Chimica Acta* 2007; **586**: 122-129.

13 Grange AH, Sovocool W. Identification of compounds in water above a pollutant plume by high-resolution mass spectrometry. *Environmental Forensics* 2007; **8**: 391-404.

14 Grange AH, Genicola FA, Sovocool W. Utility of three types of mass spectrometers for determining elemental compositions of ions formed from chromatographically separated compounds. *Rapid Communications in Mass Spectrometry* 2002; **16**: 2356-2369.

15 Santos FJ, Galceran MT. Modern developments in gas chromatography-mass spectrometry-based environmental analysis. *Journal of Chromatography A* 2003; **1000**: 125-151.

16 Portolés T, Pitarch E, López FJ, Sancho JV, Hernández F. Methodical approach for the use of GC-TOF MS for screening and confirmation of organic pollutants in environmental water. *Journal of Mass Spectrometry* 2007; **42**: 1175-1185.

17 Hernandez F, Portoles T, Pitarch E, Lopez FJ. Target and nontarget screening of organic micropollutants in water by solid-phase microextraction combined with gas chromatography/high-resolution time-of-flight mass spectrometry. *Analytical Chemistry* 2007; **79**: 9494-9504.

18 Dalluge J, Roose P, Brinkman UAT. Evaluation of a high-resolution time-of-flight mass spectrometer for the gas chromatographic determination of selected environmental contaminants. *Journal of Chromatography A* 2002; **970**: 213-223.

19 Čajka T, Hajšlova J, Kazda R, Poustka J. Challenges of gas chromatography - High-resolution time-of-flight mass spectrometry for simultaneous analysis of polybrominated diphenyl ethers and other halogenated persistent organic pollutants in environmental samples. *Journal of Separation Science* 2005; **28**: 601-611.

20 Kazda R, Hajšlova J, Poustka J, Čajka T. Determination of polybrominated diphenyl ethers in human milk samples in the Czech Republic: Comparative study of negative chemical ionisation mass spectrometry and time-of-flight high-resolution mass spectrometry. *Analytica Chimica Acta* 2004; **520**: 237-243.

21 Fay LB, Newton A, Simian H, Robert F, Douce D, Hancock P, Green M, Blank M. Potential of gas chromatography-orthogonal acceleration time-of-flight mass spectrometry (GC-oaTOFMS) in flavor research. *Journal of Agricultural and Food Chemistry* 2003; **51**: 2708-2713.

22 Leandro CC, Hancock P, Fussell RJ, Keely BJ. Quantification and screening of pesticide residues in food by gas chromatography-exact mass time-of-flight mass spectrometry. *Journal of Chromatography A* 2007; **1166**: 152-162.

23 Čajka T, Hajslova J. Gas chromatography-time-of-flight mass spectrometry in food analysis. *LC GC Europe.* 2007; **20**, 25.

24 Ibáñez M, Sancho JV, Hernández F, McMillan D, Rao, R. Rapid non-target screening of organic pollutants in water by ultraperformance liquid chromatography coupled to time-of-light mass spectrometry. *TrAC, Trends Anal. Chem.* 2008; **27**: 481-489.

25 European Comission Decision 2002/657/EC, implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Off.J.Eur. Commun. 2002.

26 Pitarch E, Medina C, Portolés T, López FJ, Hernández F. Determination of priority organic micro-pollutants in water by gas chromatography coupled to triple quadrupole mass spectrometry. *Analytica Chimica Acta* 2007; **583**: 246-258.

27 Hernández F, Ibáñez M, Sancho JV, Pozo OJ. Comparison of different mass spectrometric techniques combined with liquid chromatography for confirmation of pesticides in environmental water based on the use of identification points. *Analytical Chemistry* 2004; **76**: 4349-4357.

28 Hernández F, Sancho JV, Pozo OJ. Critical review of the application of liquid chromatography/mass spectrometry to the determination of pesticide residues in biological samples. *Analytical and Bioanalytical Chemistry* 2005; **382**: 934-946.

29 Tombesi NB, Freije H. Application of solid-phase microextraction combined with gas chromatography-mass spectrometry to the determination of butylated hydroxytoluene in bottled drinking water. *Journal of Chromatography A* 2002; **963**: 179-183.

30 Fries E, Püttmann W. Analysis of the antioxidant butylated hydroxytoluene (BHT) in water by means of solid phase extraction combined with GC/MS. *Water Research* 2002; **36**: 2319-2327.

31 Castillo M, Barceló D. Characterisation of organic pollutants in textile wastewaters and landfill leachate by using toxicity-based fractionation methods followed by liquid and gas chromatography coupled to mass spectrometric detection. *Analytica Chimica Acta* 2001; **426**: 253-264.

32 Sanches-Silva A, Cruz JM, Sendón-Garcia R, Paseiro-Losada P. Determination of butylated hydroxytoluene in food samples by high-performance liquid chromatography with ultraviolet detection and gas chromatography/mass spectrometry. *Journal of AOAC International* 2007; **90**: 277-283.

33 Soubra L, Sarkis D, Hilan C, Verger P. Dietary exposure of children and teenagers to benzoates, sulphites, butylhydroxyanisol (BHA) and butylhydroxytoluen (BHT) in Beirut (Lebanon). *Regulatory Toxicology and Pharmacology* 2007; **47**: 68-77.

34 Yang MH, Lin HJ, Choong YM. A rapid gas chromatographic method for direct determination of BHA, BHT and TBHQ in edible oils and fats. *Food Research International* 2002; **35**: 627-633.

35 Conacher HBS, Iverson F, Lau PY, Page BD. Levels of BHA and BHT in human and animal adipose tissue: Interspecies extrapolation. *Food and Chemical Toxicology* 1986; **24**: 1159-1162.

36 Oros DR, Jarman WM, Lowe T, David N, Lowe S, Davis JA. Surveillance for previously unmonitored organic contaminants in the San Francisco Estuary. *Marine Pollution Bulletin* 2003; **46**: 1102-1110.

37 Soliman MA, Pedersen JA, Park H, Castaneda-Jimenez A, Stenstrom MK, Suffet IH. Human pharmaceuticals, antioxidants, and plasticizers in wastewater treatment plant and water reclamation plant effluents. *Water Environment Research* 2007; **79**: 156-167.

38 Pedersen JA, Yeager MA, Suffet IH. Xenobiotic organic compounds in runoff from fields irrigated with treated wastewater. *Journal of Agricultural and Food Chemistry* 2003; **51**: 1360-1372.

39 Perez-Cadahia B, Laffon B, Pasaro E, Mendez J. Evaluation of PAH bioaccumulation and DNA damage in mussels (Mytilus galloprovincialis) exposed to spilled Prestige crude oil. *Comparative Biochemistry and Physiology – C Toxicology and Pharmacology* 2004; **138**: 453-460.

40 Niessner G, Klampfl CW. Direct comparison of solid-phase extraction and solid-phase microextraction for the gas chromatographic determination of dibenzylamine in artificial saliva leachates from baby bottle teats. *Analytica Chimica Acta* 2000; **414**: 133-140.

41 Calvo MB. Pedraz JL, Domínguez-Gil A. Determination of low concentrations of dibenzylamine in human plasma and urine by gas chromatography with a nitrogen-phosphorus detector. *Journal of Chromatography- Biomedical Applications* 1989; **490**: 206-212.

Table 1. Compounds detected in the GC-MS analysis of adipose human breast tissue samples

Pre-Target compounds (28 samples analyzed)	**nº of positives by GC-(QqQ)MS/MS**	**nº of positives by GC-TOF MS (target screening)**	**nº of positives by GC-TOF MS (non-target screening)[1]**
HCB	25	24	6
β-HCH	19	18	1
p,p'-DDE	27	27	12
p,p'-DDD	12	0	0
p,p'-DDT	5	0	0
oxychlordane	1	0	0
trans-nonachlor	4	1	0
mirex	2	0	0
PCB 28	3	2	0
PCB 101	5	0	0
PCB 118	10	4	0
PCB 153	25	24	4
PCB 138	23	17	8
PCB 180	26	17	2
Post-Target compounds [2] (42 samples analyzed)			
naphthalene	-	10	4
phenanthrene	-	8	0
fluoranthene	-	8	0
Pyrene	-	19	0
PCB 114	-	9	0
PCB 123	-	12	0
PCB 156	-	8	0
PCB 157	-	16	0
PCB 167	-	6	0
PCB 189	-	3	1
Non-target compounds [2] (42 samples analyzed)			
BHT	-	-	31
BHT-CHO [3]	-	-	1
PCB 4Cl	-	-	2
PCB 5Cl	-	-	1
PCB 7Cl (isomer 1)	-	-	11
PCB 7Cl (isomer 2)	-	-	2
PCB 7Cl (isomer 3)	-	-	7
PCB 8Cl	-	-	3
1,2-dimethylnaphthalene	-	-	1
2-methyl naphthalene	-	-	2
N-BBSA [3]	-	-	1

[1] Found when treating all compounds as unknown., i.e. applying the software for components detection and peak deconvolution
[2] Not included in GC-QqQ analysis
[3] Detected in the most polar HPLC-fraction (clean-up procedure B).