Journal of Visualized Experiments

Extraction of organochlorine pesticides adsorbed on plastic pellets using a pressurized fluid extractor and plastic type determination

--Manuscript Draft--

Manuscript Number:	JoVE55531R2
Full Title:	Extraction of organochlorine pesticides adsorbed on plastic pellets using a pressurized fluid extractor and plastic type determination
Article Type:	Invited Methods Article - Author Produced Video
Keywords:	Microplastics; resin pellets; pesticides; POPs; OCPs; DDT; Endosulfan; HCH; lindane; FT-IR; pressurized fluid extractor
Manuscript Classifications:	4.27.888.284: Environmental Pollutants; 94.45.12: environmental monitoring; 94.45.13: environmental pollution
Corresponding Author:	Marilyne Pflieger University of Nova Gorica Nova Gorica, SLOVENIA
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	marilyne.pflieger@ung.si
Corresponding Author's Institution:	University of Nova Gorica
Corresponding Author's Secondary Institution:	
First Author:	Marilyne Pflieger
First Author Secondary Information:	
Other Authors:	Petra Makorič
	Manca Kovač Viršek
	Špela Koren
Order of Authors Secondary Information:	
Abstract:	Plastic resin pellets, categorized as microplastics (≤ 5 mm in diameter), are small granules that can be unintentionally released to the environment during manufacturing and transport. Because of their environmental persistence, they are widely distributed in the oceans and on beaches all over the world. They can act as a vector of potentially toxic organic compounds (e.g. polychlorinated biphenyls) and might consequently negatively affect marine organisms. Their possible impacts along the food chain are not yet well understood. In order to assess the hazards associated with the occurrence of plastic pellets in the marine environment, it is necessary to develop methodologies allowing rapid determination of associated organic contaminant levels. The present protocol describes the different steps required for sampling resin pellets, analyzing adsorbed organochlorine pesticides (OCPs) and identifying the plastic type. The focus is on the extraction of OCPs from plastic pellets by means of a pressurized fluid extractor and on the polymer chemical analysis applying FT-IR spectroscopy. The developed methodology focuses on 11 OCPs and related compounds including dichlorodiphenyltrichloroethane (DDT) and its two main metabolites, lindane and two production isomers, as well as the two biologically active isomers of technical endosulfan. This protocol constitutes a simple and rapid alternative to existing methodology for evaluating the concentration of organic contaminants adsorbed on plastic pieces.
Author Comments:	
Additional Information:	
Question	**Response**

If this article needs to be "in-press" by a certain date, please indicate the date below and explain in your cover letter.	

Dear Editor,

Please find enclosed our manuscript entitled " Extraction of organochlorine pesticides adsorbed on plastic pellets using a pressurized fluid extractor and plastic type determination" that we would like to be considered for publication in Journal of Visualized Experiments.

This paper highlights a protocol for assessing the levels of organochlorine pesticides adsorbed on plastic resin pellets found on coastal areas. The chemical analysis of the polymer is addressed, as well. The focus is given to pressurized fluid extraction and ATR FT-IR spectroscopy techniques.

We consider of value publishing these data in Journal of Visualized Experiments, as they provide a simple and rapid alternative for extracting the organic contaminants from the plastic matrices and identifying the polymer constituting the resin pellets.

The techniques presented in this paper and demonstrated in video format will be highly useful for researchers working in the field of microplastics.

Maryline Pflieger and Špela Koren designed the procedures described in the manuscript.
Petra Makorič and Manca Kovač Viršek performed the experiments and analyzed the data. Finally, Maryline Pflieger wrote the manuscript.

During the preparation and submission of this manuscript, we have been kindly assisted by Benjamin Werth.

Thank you for your consideration of this manuscript. We look forward to hearing from you.

Sincerely yours,

dr. Marilyne Pflieger

TITLE:
Extraction of Organochlorine Pesticides Adsorbed on Plastic Pellets Using a Pressurized Fluid Extractor and Plastic Type Determination

AUTHORS & AFFILIATIONS:
Maryline Pflieger[1], Petra Makorič[1], Manca Kovač Viršek[2], Špela Koren[2]

[1]Laboratory for Environmental Research, University of Nova Gorica, Nova Gorica, Slovenia
[2]Sector for Marine Waters, Institute for Water of the Republic of Slovenia, Ljubljana, Slovenia

AUTHOR EMAILS:
Maryline Pflieger, (maryline.pflieger@gmail.com)
Petra Makorič, (petra.makoric@ung.si)
Manca Kovač Viršek, (manca.virsek@izvrs.si)
Špela Koren, (spela.koren@izvrs.si)

CORRESPONDING AUTHOR:
Maryline Pflieger (maryline.pflieger@gmail.com)

KEYWORDS:
Microplastics, resin pellets, pesticides, persistent organic pollutants, organochlorine pesticides, dichlorodiphenyltrichloroethane, endosulfan, hexachlorocyclohexane, lindane, Fourier transform infrared spectroscopy, pressurized fluid extractor.

SHORT ABSTRACT:
Microplastics act as vector of potentially toxic organic contaminants with unpredictable effects. This protocol describes an alternative methodology for assessing the levels of organochlorine pesticides adsorbed on plastic pellets and identifying the polymer chemical structure. The focus is on pressurized fluid extraction and attenuated total reflectance Fourier transform infrared spectroscopy.

LONG ABSTRACT:
Plastic resin pellets, categorized as microplastics (≤ 5 mm in diameter), are small granules that can be unintentionally released to the environment during manufacturing and transport. Because of their environmental persistence, they are widely distributed in the oceans and on beaches all over the world. They can act as a vector of potentially toxic organic compounds (*e.g.,* polychlorinated biphenyls) and might consequently negatively affect marine organisms. Their possible impacts along the food chain are not yet well understood. In order to assess the hazards associated with the occurrence of plastic pellets in the marine environment, it is necessary to develop methodologies that allow for rapid determination of associated organic contaminant levels. The present protocol describes the different steps required for sampling resin pellets, analyzing adsorbed organochlorine pesticides (OCPs) and identifying the plastic type. The focus is on the extraction of OCPs from plastic pellets by means of a pressurized fluid extractor (PFE) and on the polymer chemical analysis applying Fourier Transform-InfraRed (FT-

IR) spectroscopy. The developed methodology focuses on 11 OCPs and related compounds, including dichlorodiphenyltrichloroethane (DDT) and its two main metabolites, lindane and two production isomers, as well as the two biologically active isomers of technical endosulfan. This protocol constitutes a simple and rapid alternative to existing methodology for evaluating the concentration of organic contaminants adsorbed on plastic pieces.

INTRODUCTION:

Global production of plastics is continuously rising since the 1950's to reach 311 million tons in 2014 with about 40% used in packaging[1]. In parallel, increasing quantities of these materials are accumulating in the environment, which might pose a serious threat to the ecosystems[2]. Although already reported in the 1970's, the occurrence of plastic debris in the marine environment has only received a greater attention in the past decade. Especially microplastics, plastic fragments with a diameter of ≤ 5 mm, are now recognized as one of the main marine water quality issues[3].

Plastic resin pellets are small granules generally in the shape of a cylinder or a disk and with a diameter of a few mm (*e.g.,* 2 to 5 mm)[4,5]. They fall in the category of microplastics. These plastic granules are industrial raw material from which final plastic products are manufactured through re-melting and molding at high temperature[6]. They can be unintentionally released to the environment during manufacturing and transport. For instance, they can be directly introduced to the ocean through accidental spills during shipping[4,7,8]. They can be carried from land to oceans by surface run-off, streams and rivers. Because of their environmental persistence, plastic pellets are widely distributed in the oceans and found on beaches all over the world[4]. They can negatively affect marine organisms and can enter the food chain, where their effects are unpredictable[6,7]. Furthermore, several studies have revealed the presence of environmental contaminants adsorbed onto plastic pellets collected in a coastal environment, which act as vector of these potentially toxic chemicals[4,9,10]. In fact, there is laboratory evidence suggesting that these chemicals can bioaccumulate in tissues of organisms after being released from ingested plastic fragments[11,12].

In order to better assess the hazards associated with the occurrence of plastic pellets in the marine environment, it is necessary to develop methodologies that can determine sorbed organic contaminants. An important step is the extraction of the chemicals from the plastic matrices, which can present heterogeneous physical-chemical characteristics depending on the polymer type, its degradation stage, and pre-treatments. Most of the investigations reported in the literature use maceration or Soxhlet techniques[4,5,6,9,13,14,15,16,17,18], which are solvent and/or time consuming. Regarding the growing interest for this issue, alternatives should be developed, for a faster evaluation of organic contaminants adsorbed on plastic pieces. In addition, plastic chemical analysis provides information about the chemical structure of the microplastics. As a result, the predominant types of polymers and copolymers present in the environment can be evaluated. Although plastic fragments are usually made of polyethylene (PE) and polypropylene (PP)[5], some sampling locations can present a particular profile where other categories are significantly represented (*e.g.,* ethylene/vinyl acetate copolymer and polystyrene (PS)). FT-IR spectroscopy is a reliable and user-friendly technique for polymer

identification commonly used to identify microplastics[19,20].

The main aim of the present work is to offer a rapid and simple option for extracting OCPs and related compounds from plastic pellets by means of a PFE. However, the design of the protocol includes all steps leading to the determination of sorbed OCPs, from the sampling of the resin pellets to the analysis of the compounds. The method of identifying the plastic type is also described. The developed methodology focuses on 11 OCPs and related compounds: i) DDT (2,4'- and 4,4'-dichlorodiphenyltrichloroethane) and its two main metabolites DDE (2,4'- and 4,4'-dichlorodiphenyldichloroethylene) and DDD (2,4'- and 4,4'-dichlorodiphenyldichloroethane); ii) the isomer gamma-hexachlorocyclohexane (γ-HCH) as the main ingredient of the pesticide lindane and the two isomers α-HCH and β-HCH released during its production[15]; iii) and the two biologically active isomers endosulfan I (Endo I) and II (Endo II) present in the technical endosulfan. The studied pesticides are broad-spectrum insecticides, chemically stable, hydrophobic, and classified as persistent organic pollutants (POPs) by the Stockholm Convention[21].

PROTOCOL:

1. Plastic Pellet Sampling

1.1. Before going to the field, triple rinse all required sampling materials (*e.g.,* tweezers and aluminum foil) with acetone or ethanol (99%). In case the material cannot be solvent-rinsed, heat it at 450 °C overnight in an oven (*e.g.,* glassware).

Note: In tourist areas, obtain information about possible beach cleaning activities that would remove most of the marine litter including microplastics. If possible, plan the sampling ahead of this operation. If sampling during the clean-up season, specify the details of this activity in the identity form (*e.g.,* dates, clean-up method used, *etc.*)

1.2. Wearing gloves, collect plastic pellets from the beach with solvent-rinsed stainless steel tweezers.

1.3. Sample 50 to 100 pellets per location, which corresponds to 5 to 10 replicates per location (10 pellets per replicate). If the required number of pellets cannot be obtained, collect the maximum pellets possible and specify it in the identity beach form.

1.4. At the end of the sampling, wrap the collected pellets in solvent-rinsed aluminum foil. Glass bottles can be used as an alternative or even paper bags.

1.5. Fill in the identity form of the selected beach with the missing information (*i.e.,* beach location, weather conditions, details on pellets, *etc.*).

1.6. Transport the samples to the laboratory in an icebox if the ambient temperature exceeds 25 °C. This step can be skipped in the case of short trips (*e.g.,* < 1 h).

1.7. Once in the laboratory, gently wipe off removable particles (*e.g.,* sand) of the pellets. Dry the samples if necessary in a desiccator prior to storage (darkness, T < 25 °C). Avoid rooms where OCPs might be in use (*e.g.,* storage of standard solutions).

1.8. Store the pellets in the fridge (4 °C) for short periods (*i.e.,* few days) or in the freezer (-18 °C) for longer periods in solvent-rinsed aluminum foil.

1.9. Avoid exposure of samples to artificial light or sunlight. Handle the samples as little as possible before analysis to decrease the risk of contamination.

2. Extraction of OCPs from Plastic Pellets

2.1. To reduce the risk of contamination, work in a clean laboratory using carefully washed glassware as follows: 2 rinses with analytical-grade acetone, dichloromethane and n-hexane. Dry the glassware under nitrogen flow and protect from contact with ambient air (*e.g.,* cover with cleaned aluminum foil). Apply this cleaning procedure in the further steps of the protocol (*i.e.,* sections 3 and 4).

2.2. Using solvent-rinsed tweezers, sort the pellets by color in the following categories: white/transparent, whitish/yellowish, yellow/orange, amber/brown, and pigmented (*e.g.,* red, green, blue, *etc.*)

2.3. Gather 10 pellets of similar color randomly (*i.e.,* plastic type not considered), which will constitute one replicate.

2.4. Weigh the sample on an analytical balance and record the mass. At this stage, the samples can be put back in the fridge or freezer.

2.5. To take into account the background contamination, perform a blank sample with each set of replicates (*e.g.,* 1 blank for 5 replicates). To this end, apply the same protocol as described above, but do not add plastic pellets in the extraction cell. This blank sample will undergo the further steps of the protocol and be analyzed together with the samples.

2.6. Switch on the PFE. Download the extraction method and warm-up the instrument to 60 °C. The details of the method are as follows:

2.6.1. Set the temperature to 60 °C and the pressure to 100 bar.

2.6.2. Select one cycle with heat-up time of 1 min, a hold time of 25 min, and a discharge time of 2 min.

2.6.3. Set the solvent and gas (N_2) flush times to 3 min each.

2.6.4. Select n-hexane as the extraction solvent.

2.7. While the instrument is warming up, prepare the extraction cell as described below. If necessary, adapt the protocol to the supplier's instructions of your instrument:

2.7.1. Place the bottom filter and the frit in the extraction cell. Close it and turn it over.

2.7.2. Fill approximately half the cell with cleaned quartz sand using a funnel.

2.7.3. Add the weighed sample (*i.e.,* one replicate of 10 pellets). Frozen plastic pellets should be placed in the fridge overnight prior to extraction.

2.7.4. Add quartz sand up to 1 cm from the top of the cell. Take special care to use ultra clean quartz sand (or alternatively glass beads) since it is exposed to the same extraction conditions as the samples. To clean the sand, successively extract it in the PFE in analytical-grade dichloromethane and n-hexane, applying 2 or more cycles per solvent (*e.g.,* 30 min at 100 °C under 100 bar). Alternatively, use an ultrasonic bath and/or rotating evaporator. Repeat the cleaning procedure, if necessary.

2.7.5. Insert the top filter in the cell and place the cell in the instrument.

2.8. Place the collecting vessels in the instrument and start the extraction method (total run of about 35 min).

2.9. When the method is completed, empty the extraction cell in a cleaned glass vessel (*e.g.,* beaker, glass cell-culture dish) and retrieve the 10 pellets in the sand. Store them in a container until further analysis for plastic identification (*e.g.,* zip bag or glass vial).

3. Concentration and Clean-up of the Extract

3.1. Transfer the obtained extract (about 40 mL) from the collecting vessel to a glass tube and evaporate it to 1 mL in a rotating concentrator set to 35 °C for 20 min. Alternative methods could be used such as evaporation under nitrogen flow or rotating evaporator. The temperature and duration should be optimized accordingly.

3.2. In the meantime, prepare the solid-phase extractor (SPE) by placing a waste tube in the rack and a cartridge filled with activated magnesium silicate sorbent (1 g) on the manifold in the close-valve position. The clean-up is based on the EPA method 3620C[22] as follows:

3.2.1. Turn the vacuum on at the source and add 4 mL of hexane in the cartridge to activate the sorbent.

3.2.2. Open the valve and let the solvent pass through the entire sorbent bed. Then, close the valve and allow the sorbent to soak in hexane for 5 min.

3.2.3. Open the valve and let the solvent pass through, but close the valve before the sorbent dries off.

3.2.4. When the sample is concentrated, transfer it to the cartridge with a glass Pasteur pipette. Gently open the valve and let it pass through slowly. 1–2 drops per second is an appropriate speed.

3.2.5. Rinse the glass tube containing the extract with 0.5 mL of hexane and add it to the cartridge when the extract has passed through.

3.2.6. When the entire solvent has passed through, close the valve and turn off the vacuum.

3.2.7. Replace the waste tube with a collecting tube and use a clean solvent guide needle.

3.2.8. Add 9 mL of acetone/hexane (10/90, v/v) to the cartridge and turn on the vacuum at the source. Allow the sorbent to soak in the solvent for 1 min.

3.2.9. Open the valve and collect the entire eluate in the collecting tube.

3.3. Place the collecting tube in the concentrator and evaporate the solvent for 9 min at 35 °C in order to reach 1 mL of eluate.

3.4. Transfer the concentrated eluate into an amber autosampler vial with a glass Pasteur pipette. At this stage, the samples can be stored in the freezer prior to analysis.

4. Analysis of the Cleaned and Concentrated Extract

4.1. Download the analytical method on the control software of the GC-μECD instrument (gas chromatograph equipped with a micro electron capture detector). The details of the method are as follows:

4.1.1. Set the injector to splitless mode, its temperature to 250 °C, and the purge time to 1 min.

4.1.2. Set the flow of the carrier gas (He) to 1.5 mL min^{-1}.

4.1.3. Program the column oven with the following temperature gradient: 60 °C hold for 1 min, ramp of 30 °C min^{-1} to 200 °C, ramp of 5 °C min^{-1} to 230 °C, ramp of 3 °C min^{-1} to reach 250 °C, hold this temperature for 5 min.

4.1.4. Set the detector temperature to 300 °C and the back-up gas flow (N_2) to 60 mL min^{-1}.

4.2. Place the vial containing the sample (cleaned and concentrated) in the autosampler rack and run the method (run time of 23.3 min). Inject 2 μL of sample.

4.3. After the analysis, identify the different compounds on the chromatogram by their retention times and record the corresponding peak areas.

4.4. Taking into account the recoveries (R) and the peak areas (A_1), calculate the concentration (C_1) of each OCP in the extract using the equations of the calibration curves as follows:

$$C_1 = \frac{\left[\left(\frac{A_{1\times}100}{R}\right) - b\right]}{a}$$

where *b* is the intercept at the origin and *a* is the slope of the calibration equation,

$$C = ax + b.$$

4.5. Taking into account the mass (m) of the replicate (*i.e.,* 10 pellets; see section 2.4) and the volume (V) of the final extract (*i.e.,* 1 mL), calculate the concentration (C_2) of each OCP adsorbed on the plastic pellets (*i.e.,* ng of OCP per g of plastic pellet):

$$C_2 = \frac{V \times 10^{-3} \times C_1}{m}$$

5. Plastic Type Identification

5.1. Transfer the pellets in a glass Petri dish and place it in a plastic bag.

5.2. Hold one pellet with tweezers and cut a slice of the pellet with a scalpel. The plastic bag prevents the loss of pellets during the cutting process.

5.3. Clean the attenuated total reflectance (ATR) crystal of the FT-IR instrument with ethanol.

5.4. Record a background spectrum.

5.5. Place the fragment on the ATR crystal and screw the sample holder. The inner side of the piece must be in contact with the crystal.

5.6. Scan the sample and record the spectrum.

5.7. Identify the polymer constituting the plastic pellet by comparing the obtained spectrum to a spectra library. Although more time-consuming, the interpretation of the obtained spectra could be carried out manually as well, but most probably without reaching the degree of specificity achieved with a library search.

REPRESENTATIVE RESULTS:

Plastic pellets are usually found along the high and low tide lines of sandy beaches (**Figure 1A**).

They can also stick to seagrass freshly stranded on beaches, after a storm for instance. They can occasionally be found on pebble and stony beaches in accumulation areas of stranded material. Plastic pellets are usually easily recognizable by their shape, size and color as shown in **Figure 1B** (see the two middle columns). They could be mistaken for tiny gravels (see columns 5 and 6), small biological fragments, or particles of different origins (see columns 1 and 2). Once in the laboratory, the suspicious items can be discarded. In case of doubt, it is possible to check the floatability of the samples in double distilled water. Gravels will sink whereas plastic pellets will mostly float. A sample of plastic pellets collected on a beach is shown in **Figure 1C** for illustration. An example of the identity beach form to be filled in the field is provided **Figure 1D**.

[place figure 1 here]

Whitish and yellowish pellets are usually predominant over other pellets, especially pigmented ones. However, some sampling sites present a particular profile and therefore it is advised to classify the plastic pieces by color (visual assessment) prior to extraction. A visual reference can be created to help sorting the pellets as presented on **Figure 2** (from left to right: white/transparent, whitish/yellowish, yellow/orange, amber/brown, and pigmented).

[place figure 2 here]

It can happen that some pellets start to melt during the extraction process. Thus, quartz sand particles will stick on their surface after extraction. For instance, in **Figure 3A,** the isolated pellet on the left of the Petri dish has sand particles sticking on its surface due to melting. This often occurs with ethylene/vinyl acetate copolymers due to their low melting point compared to other plastic polymers such as PE and PP. Exceptionally, the melting process can be too severe and the extract will appear milky (**Figure 3B**). In this case, it is advised to discard the sample immediately after extraction. This extract would clog the sorbent of the SPE cartridge.

[place figure 3 here]

As a first approach, spiked virgin pellets were prepared in order to optimize the extraction step and assess its repeatability. As can be seen from **Figure 4A**, all 11 OCPs were extracted applying the described protocol. In addition, **Figure 4B** illustrates the analysis result of OCPs extracted from pellets sampled on a beach at the Adriatic coast. In this case, 8 OCPs out of 11 were detected. The chromatographic peak identification is based on the retention times obtained from the injection of OCPs individual standard solutions. A deviation from standard peak retention time of 0.1% is accepted as the maximum. The calculation of OCPs concentrations is based on the analysis of standard solutions. Calibration equations and recoveries from the SPE and concentration steps must be determined for each studied compound prior to sample analysis (**Table 1**).

[place figure 4 and Table 1 here]

A representative concentration is determined for each sampling location by analyzing at least 3

replicates of 10 pellets and taking the median value. The latter is preferred to the average value due to the dispersion of the results[4]. **Figure 5** shows an example of results based on 5 replicates.

[place figure 5 here]

The chemical analysis of the plastic is carried out on an ATR-FT-IR spectrometer. The measurement is performed on the inner side of a pellet slice. Plastic pieces are covered by biofilms and/or by adherent layer(s), which can interfere in the IR spectra of the sample. Thus, cutting the pellets allows an easier identification of the polymer than processing uncut items, because the ATR crystal is in contact with less contaminated material. The chemical composition analysis results of an uncut pellet are shown in **Figure 6A**. The item was identified as rubber with a probability of about 66% at the highest. **Figure 6B** presents the results obtained from a slice of the same pellet, which was ultimately shown to be made of PE with a probability of 99%. The second measurement was performed on the inner side of the fragment.

[place figure 6 here]

PE, as identified in **Figure 7A**, is the most common polymer type found in plastic pellets, followed by PP (**Figure 7B**). Ethylene/vinyl acetate copolymer is the 3rd most common plastic type usually identified (**Figure 7C**). Pellets made of PS can occasionally also be found (**Figure 7D**). An example of plastic type identification for a replicate of 10 pellets is given in **Table 2**. As can been seen, the sample consists of 70% by PE.

[place figure 7 and Table 2 here]

FIGURE AND TABLE LEGENDS:

Figure 1: (**A**) Plastic resin pellets stranded on a sandy beach at the tide line. (**B**) Plastic resin pellets vs. gravel and other stranded materials. Fragments of different origins are presented in the 1st and 2nd columns from the left. Small gravel particles are aligned in columns 5 and 6. Plastic pellets are in the middle columns. (**C**) Sample of plastic resin pellets. (**D**) Example of an identity beach form.

Figure 2: Classification of plastic pellets by color, from left to right: white/transparent, whitish/yellowish, yellow/orange, amber/brown and pigmented.

Figure 3: (**A**) The isolated pellet on the left of the Petri dish has sand particles sticking on its surface due to melting. (**B**) Extraction with polymer break down. The melting process can render the extract to appear milky.

Figure 4: (**A**) Chromatogram of OCPs extracted from spiked virgin PE pellets. (**B**) Chromatogram of OCPs extracted from pellets sampled on the Adriatic coast.

Figure 5: Median concentration of OCPs extracted from pellets sampled on the Adriatic coast.

The data shows an example of results based on 5 replicates.

Figure 6: (**A**) FT-IR spectrum of the uncut pellet and best hit results from the spectra library. (**B**) FT-IR spectrum of the pellet slice and best hit results from the spectra library.

Figure 7: FT-IR spectrum and best hit results of a pellet identified as (**A**) PE (99.0%); (**B**) PP (98.9%); (**C**) ethylene/vinyl acetate copolymer (97.0%); and (**D**) PS (99.6%).

Table 1: Example of calibration and recovery results obtained for the 11 studied OCPs.

Table 2: Polymer identification results of a pool of 10 pellets**.**

DISCUSSION:

Most studies focusing on organic contaminants associated to plastic pellets have relied on classical extraction methods of the adsorbed chemicals. The Soxhlet apparatus is the most widely used technique with typical extraction times ranging from 12 to 24 hours and with high consumption of organic solvents (*i.e.,* from 100 to 250 mL per extraction)[23]. Maceration extractions require a long contact time between the sample and the organic solvent (*e.g.,* 6 days)[4] and can be hastened by adding an ultrasonication step. In contrast, pressurized fluid extraction, as described in this study, is an efficient way to rapidly extract analytes from solid or semi-solid matrices under high pressure and temperature using a reduced amount of solvent (*e.g.,* 40 mL). Although it is commonly used as an alternative to the Soxhlet method, this technique has rarely been employed in the field of microplastics[14]. One of the limitations linked to the application of this technique to the analysis of plastic fragments is the potential melting of polymers, which are then difficult to remove from the extract and often make its analysis impossible. This issue is not encountered when extracting organics from homogeneous matrices. In this case, the extraction temperature is set according to the polymer type of the plastic sample. Microplastic samples are composed of a heterogeneous mixture of items made of various polymer types in different degradation states, which often cause the early melting of the plastic. Thus, the temperature in the PFE cell must be optimized to allow the extraction of OCPs regardless of the polymer type and its degradation state. In this work, a temperature of 60 °C together with a long hold time were found to be a good compromise between extraction efficiency and melting issues. Only rubber and aged ethylene/vinyl acetate copolymer are prone to melting, but these polymers are usually present at such low amounts in the sample that they do not affect extraction.

In many studies[4,8,13,16,18], only aged PE pellets are analyzed for their adsorbed organic contaminant content. Because of their surface properties, this category of polymers has a greater affinity to adsorb environmental pollutants than other type of pellets and they are the predominant polymer class[4]. However, some sampling locations present a special profile with an abundance of less aged pellets (*i.e.,* white or transparent) and/or a higher variety in polymer types than commonly found. Thus, a different approach is suggested here to avoid a possible overestimation of the organic contaminant levels. The classification of plastic pellets is based on color rather than on polymer type. Moreover, the identification of the plastic type can still be

carried out after the extraction step. By proceeding in this order, the risk of sample contamination during the polymer chemical analysis is lowered and the plastic identification process can be facilitated by cutting the pellets, as previously explained. Extracting organic contaminants from items that are mistaken for plastic pellets would be the main limitation of this methodology. However, it can be underlined that only a negligible fraction (*i.e.,* less than 0.5%) of the sampled pellets is shown not to be made of plastic polymer after chemical analysis.

This protocol was developed for the determination of OCPs adsorbed on plastic pellets. However, it can be adapted for detection of other categories of organic contaminants usually found associated to microplastics such as polychlorinated biphenyls (PCBs) or polycyclic aromatic hydrocarbons (PAHs), as well as plastic softeners or additives. To this end, the clean-up step would have to be further optimized by eluting the sorbent with several successive solvents of different polarities[4,10]. To some extent, the extraction solvent composition could also be modified, by adding a fraction of dichloromethane and/or acetone to hexane, for instance. Finally, new analytical methods must be developed specially for the compounds to be investigated. Although Gas Chromatography-Electron Capture Detector (GC-ECD) is a sensitive technique, its selectivity for halogenated compounds limits its application to other classes of compounds. Moreover, peak identification is only based on retention times, which can lead to misinterpretation of chromatograms. To lower the risk of misidentification, a deviation from standard solution retention times of only 0.1% is accepted. Gas chromatography equipped with a mass spectrometer (GC-MS) is an appropriate technique for validating the peak identification. It could be run in parallel to GC-ECD or used as a single analysis method if its sensitivity allows the quantification of trace concentrations.

This methodology focuses on resin pellets, but it could be further optimized for the analysis of other microplastic categories. However, the sorting of plastic fragments from environmental samples (*e.g.,* sea surface, sediment, or biota) is more challenging than the one of pellets and a visual identification is not appropriate. Thus, the chemical analysis of the polymers should be performed prior to extraction. Knowing that microplastic sizes range from 5 mm to a few hundred µm (*e.g.,* 300 µm), the analysis should be performed on a micro ATR-FTIR spectrometer, which is adapted for the measurements of small particles[19,20]. Moreover, the separation of microplastics from environmental samples usually requires the use of solvents (*e.g.,* ethanol) and/or strong acids or bases (*e.g.,* acid digestion of tissues), which can desorb and/or degrade the organic contaminants associated to the particles. Thus, alternative separation techniques should be developed, which would preserve the chemicals. In addition, it should be underlined that the quantity of microplastics detected in sea surface and biota is often insufficient for performing quantitative analyses of organic compounds. This protocol is adapted to process plastic fragments visible to the naked eye and made of hard polymers. It is not likely to work on soft materials or extremely small items (*i.e.,* < 1 mm). Thus, the microplastic categories of films, filaments, and foams should be discarded from the samples. Nevertheless, small microplastic pieces could be analyzed for their organic contaminant content and their polymer type. In this case, it is advised to cut the items in small particles of a few mm prior to extraction or FT-IR analysis.

ACKNOWLEDGMENTS:
This work was funded by IPA Adriatic Cross-border Cooperation Program 2007-2013, within the DeFishGear project (1°str/00010).

DISCLOSURES:
The authors have nothing to disclose.

REFERENCES:

1. Plastic Europe. Plastics – the Facts 2015. An analysis of European plastics production, demand and waste data. Available on the website: http://www.plasticseurope.org/.
2. Wang, J., Tan, Z., Peng, J., Qiu, Q., Li, M. The behaviors of microplastics in the marine environment. *Mar Environ Res.* **113**, 7-17, DOI: 10.1016/j.marenvres.2015.10.014, (2016).
3. UNEP. Marine plastic debris and microplastics – Global lessons and research to inspire action and guide policy change. *United Nations Environment Programme*, Nairobi, Available on the website: http://www.unep.org/, (2016).
4. Ogata, Y., *et al.* International Pellet Watch: Global monitoring of persistent organic pollutants (POPs) in coastal waters. 1. Initial phase data on PCBs, DDTs, and HCHs. *Mar Pollut Bull.* **58**(10), 1437–1446, DOI:10.1016/j.marpolbul.2009.06.014 (2009).
5. Andrady, A. L. Microplastics in the marine environment. *Mar Pollut Bull.* **62**(8), 1596–1605, DOI: 10.1016/j.marpolbul.2011.05.030, (2011).
6. Antunes, J. C., Frias, J. G. L., Micaelo, A. C., Sobral, P. Resin pellets from beaches of the Portuguese coast and adsorbed persistent organic pollutants. *Estuarine Coastal Shelf Sci*. **130**, 62-69, DOI: 10.1016/j.ecss.2013.06.016, (2013).
7. Cole, M., Lindeque, P., Halsband, C., Galloway, T. S. Microplastics as contaminants in the marine environment: A review. *Mar Pollut Bull.* **62**(12), 2588–2597, DOI: 10.1016/j.marpolbul.2011.09.025 (2011).
8. Takada H. Call for pellets! International Pellet Watch Global Monitoring of POPs using beached plastic resin pellets. *Mar Pollut Bull.* **52**(12), 1547–1548, DOI: 10.1016/j.marpolbul.2006.10.010, (2006).
9. Teuten E. L. Transport and release of chemicals from plastics to the environment and to wildlife. *Phil Trans R Soc B*. **364**, 2027–2045, DOI: 10.1098/rstb.2008.0284, (2009).
10. Heskett, M., *et al*. Measurement of persistent organic pollutants (POPs) in plastic resin pellets from remote islands: Toward establishment of background concentrations for International Pellet Watch. *Mar Pollut Bull.* **64**(2), 445–448, DOI: 10.1016/j.marpolbul.2011.11.004, (2012).
11. Besseling, E., Wegner, A., Foekema, E., Van Den Heuvel-Greve, M., Koelmans, A. A. Effects of microplastic on fitness and PCB bioaccumulation by the lugworm Arenicola marina (L.). *Environ Sci Technol.* **47**(1), 593–600, DOI: 10.1021/es302763x, (2013).
12. Rochman, C. M., Hoh, E., Kurobe, T., The, S. J. Ingested plastic transfers hazardous chemicals to fish and induces hepatic stress. *Sci Rep.* ***3***, 3263, DOI: 10.1038/srep03263, (2013).
13. Endo, S., *et al.* Concentration of polychlorinated biphenyls (PCBs) in beached resin pellets: Variability among individual particles and regional differences. *Mar Pollut Bull.* **50**(10), 1103–1114, DOI: 10.1016/j.marpolbul.2005.04.030, (2005).

14. Frias, J. P. G. L., Sobral, P., Ferreira, A. M. Organic pollutants in microplastics from two beaches of the Portuguese coast. *Mar Pollut Bull.* **60**(11), 1988–1992, DOI: 10.1016/j.marpolbul.2010.07.030, (2010).
15. Karapanagioti, H. K., Endo, S., Ogata, Y., Takada, H. Diffuse pollution by persistent organic pollutants as measured in plastic pellets sampled from various beaches in Greece. *Mar Pollut Bull.* **62**(2), 312–317, DOI: 10.1016/j.marpolbul.2010.10.009, (2011).
16. Mizukawa, K., *et al*. Monitoring of a wide range of organic micropollutants on the Portuguese coast using plastic resin pellets. *Mar Pollut Bull.* **70**(1-2), 296–302, DOI: 10.1016/j.marpolbul.2013.02.008, (2013).
17. Gauquie, J., Devriese, L., Robbens, J., De Witte, B. A qualitative screening and quantitative measurement of organic contaminants on different types of marine plastic debris. *Chemosphere*. **138**, 348–356, DOI: 10.1016/j.chemosphere.2015.06.029, (2015).
18. Yeo, B. G., *et al.* POPs monitoring in Australia and New-Zealand using plastic resin pellets, and International Pellet Watch as a tool for education and raising public awareness on plastic debris and POPs. *Mar Pollut Bull.* **101**(1), 137–145, DOI: 10.1016/j.marpolbul.2015.11.006, (2015).
19. Kovač Viršek, M., Palatinus, A., Koren, Š., Peterlin, M., Horvat, P., Kržan, A. Protocol for microplastics sampling on the sea surface and sample analysis. *J Vis Exp*. (118). E55161, doi:10.3791/55161 (2016).
20. Löder, M. G. J., Kuczera, M., Mintenig, S., Lorenz, C., Gerdts, G. Focal plane array detector-based micro-Fourier-transform infrared imaging for the analysis of microplastics in environmental samples. *Environ Chem.* **12**(5), 563-581, DOI: 10.1071/EN14205, (2015).
21. Stockholm Convention on Persistent Organic Pollutants (POPs) as amended in 2009 available on the website: http://chm.pops.int/Home/tabid/2121/Default.aspx.
22. EPA - Environmental protection Agency, Method 3620C: Florisil Cleanup, part of Test Methods for Evaluating Solid Waste, Physical/Chemical Methods (2014) available on the website: https://www.epa.gov.
23. Hirai, H., *et al*. Organic micropollutants in marine plastics debris from the open ocean and remote and urban beaches. *Mar Pollut Bull.* **62**(8), 1683–1692, DOI: 10.1016/j.marpolbul.2011.06.004, (2011).

Figure 1A

Click here to download Figure Figure 1A.tif

Figure 1B

Click here to download Figure Figure 1B.tif

Figure 1C

Click here to download Figure Figure 1C.tif

Figure 1D

Click here to download Figure Figure 1D.pdf

GENERAL INFORMATION
Organisation:
Researchers:
Date of sampling:
Time of sampling:
Sample Name:
SAMPLING SITE DATA
Name of the beach:
GPS coordinates:
Beach description (sandy, pebble…):
Weather:
Air temperature:
PELLET DATA
Number of plastic pellets sampled:
Where did you find the pellets?
✓ **Along the tide line:**
✓ **In accumulation area:**
✓ **Attached to other stranded materials (seagrass…):**
✓ **Other:**
OTHER REMARKS

Figure 2

Click here to download Figure Figure 2.tif

Figure 3A

Click here to download Figure Figure 3A.tif

Figure 3B

Click here to download Figure Figure 3B.tif

Figure 5

Click here to download Figure Figure 5.tif

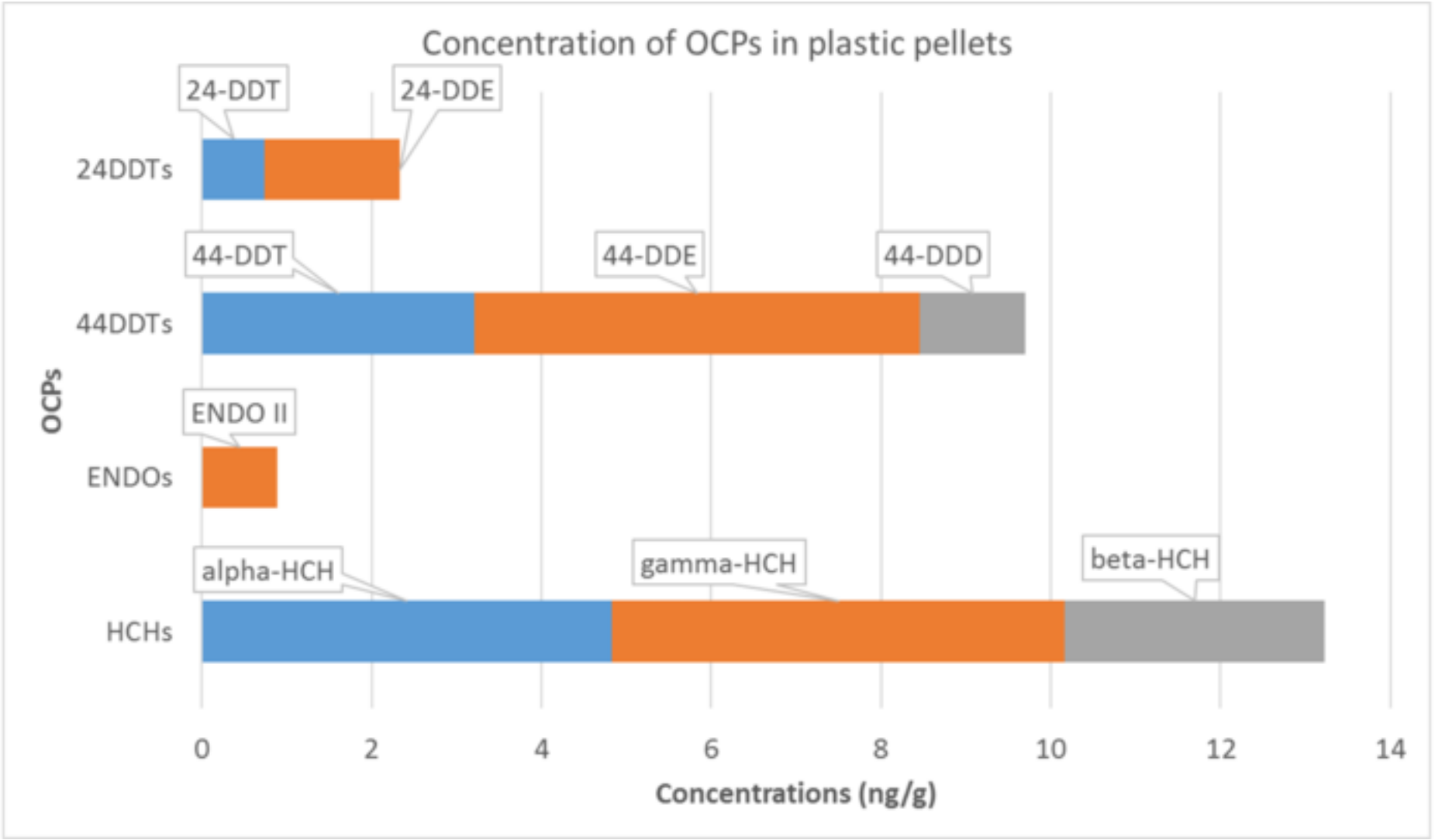


Figure 4A

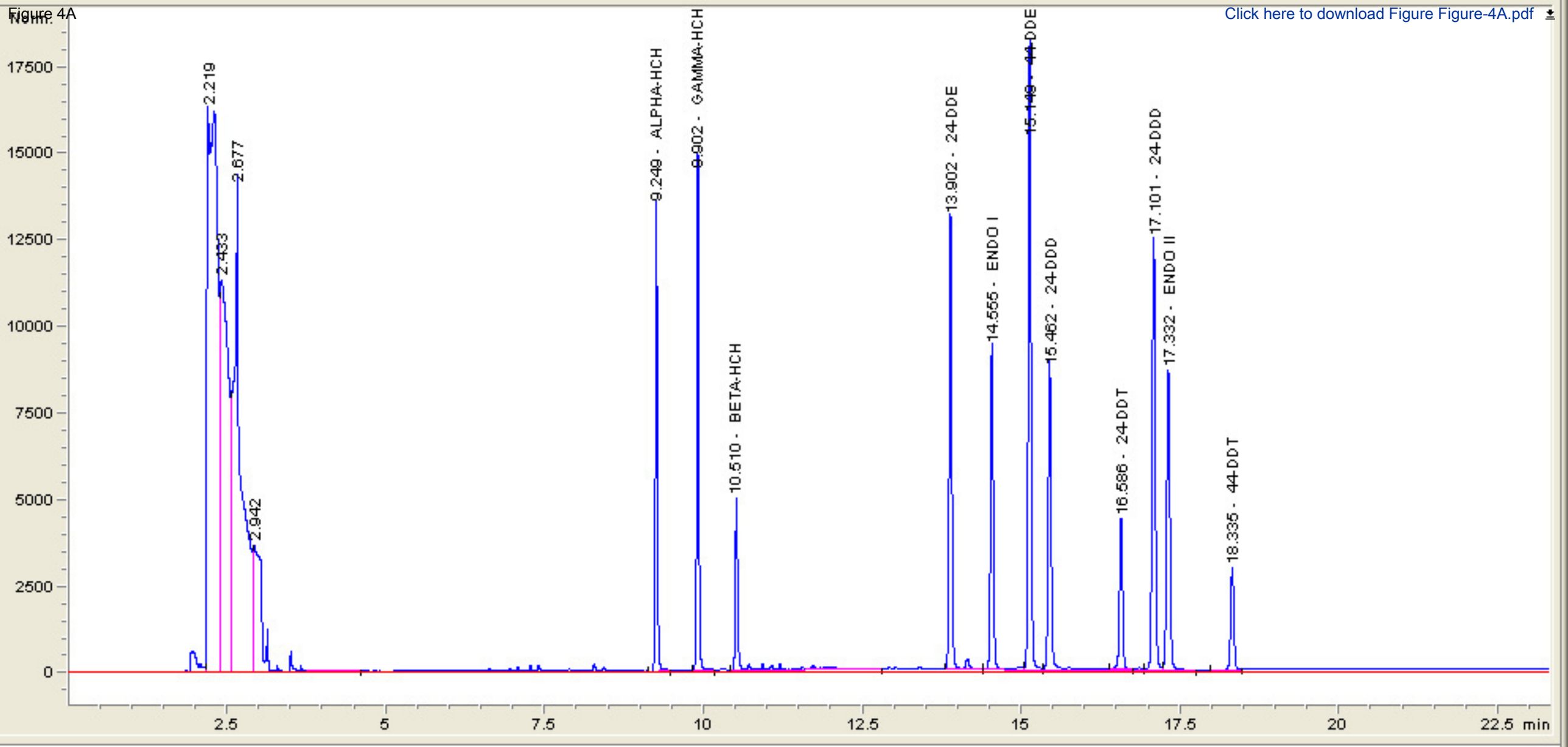


Figure 4B

Click here to download Figure Figure-4B.pdf

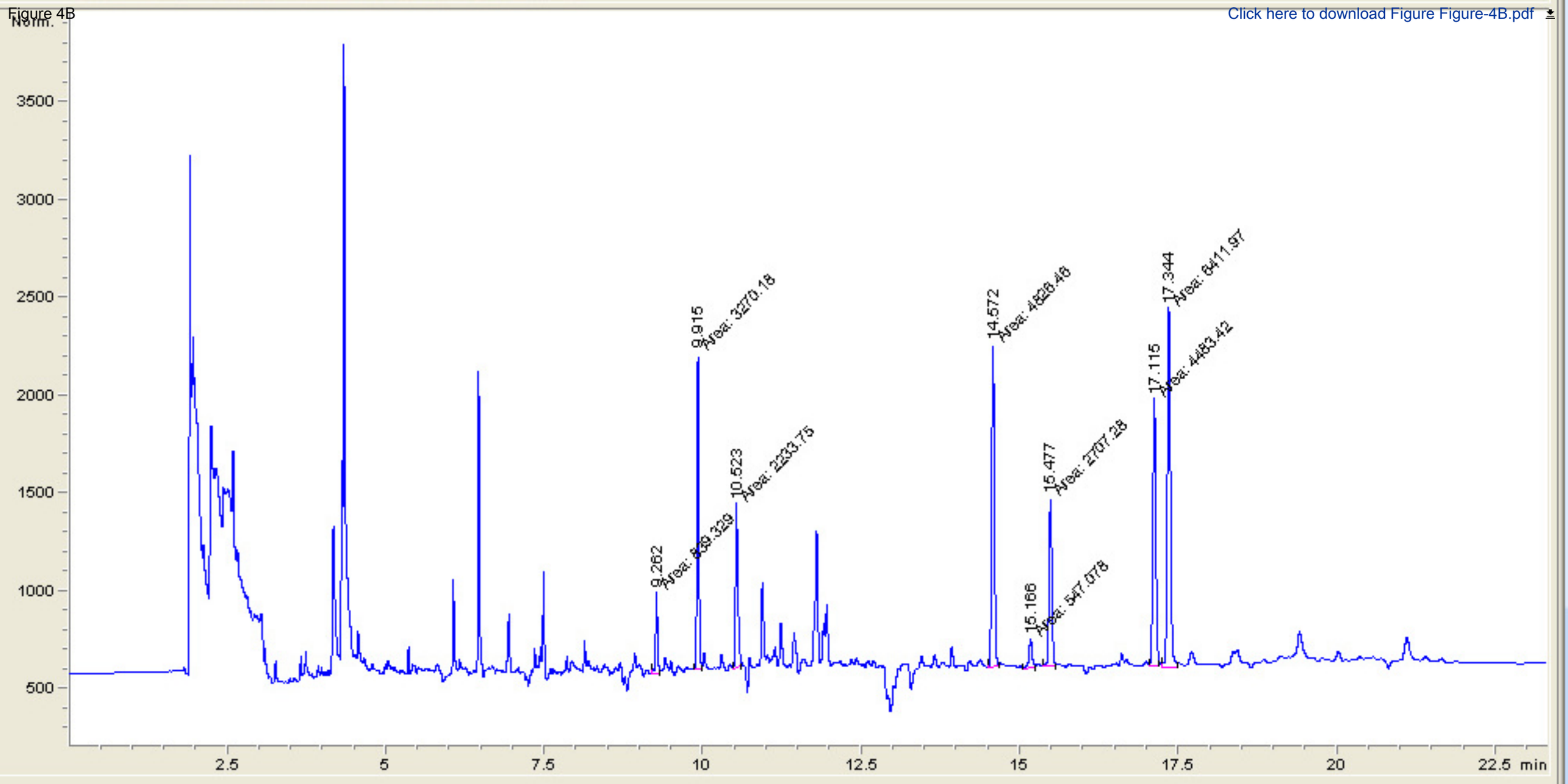


Figure 6A

Click here to download Figure Figure-6A.pdf

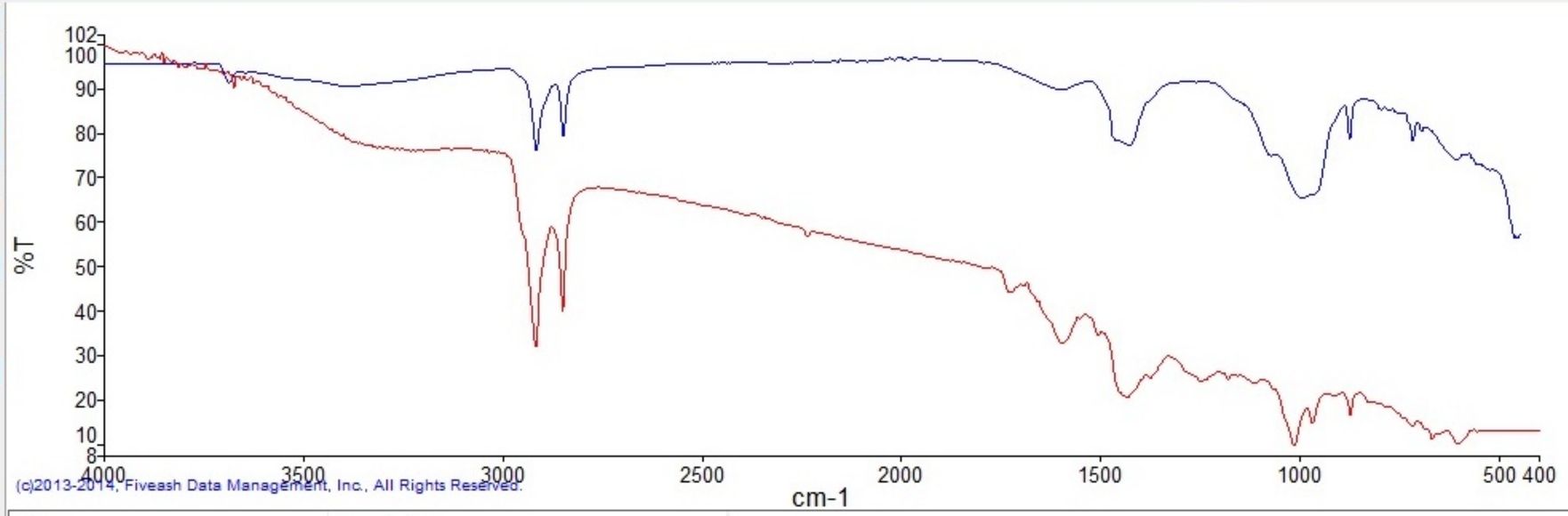
Search Spectra Search Results:

	Sample Name	Search Score	Search Best Hit	Search Best Hit Description
▶	essai2 not cut	0,664704	P01090	P1090.SP HYPALON RUBBER, 65 DUROMETER, CONTAINS TALC, GE XT...

Search Hit List

	Search Score	Search Reference Spectrum Description
1 ▶	0,664704	P1090.SP HYPALON RUBBER, 65 DUROMETER, CONTAINS TALC, GE XTAL
2	0,656278	P1108.SP NEOPRENE O-RING CORD STOCK, GE XTAL
3	0,652156	P1128.SP SUPER SOFT NEOPRENE RUBBER, 20A DUROMETER
4	0,637556	P1130.SP ULTRA STRENGTH NEOPRENE RUBBER, 50A DUROMETER
5	0,634867	P1134.SP ULTRA STRENGTH NEOPRENE RUBBER, 60A DUROMETER, GE XTAL
6	0,628792	R0152.SP REDDEVIL(R) BLACKTOP & ROOF, ASPHALT SEALANT, BLACK
7	0,625154	H561.SP PERSIL, WASHING AGENT
8	0,625033	P1086.SP HIGH STRENGTH MULTIPURPOSE NEOPRENE RUBBER, 80A DUROMETER
9	0,618574	P1076.SP HIGH STRENGTH MULTIPURPOSE NEOPRENE RUBBER, 30A DUROMETER
10	0,618152	C07901.SP TRANS-2-OCTEN-1-OL, 18409-17-1

Accept as Best Hit | Search Residuals



Name	Description
essai2 not cut	Sample 528 By mvirsek Date petek, septem...
P01090	P1090.SP HYPALON RUBBER, 65 DUROMET...

Additional properties of P01090

Property	Information
Copyright	(c)2013-2014, Fiveash Data Management, Inc., All Rights Reserved.

Figure 6B

Click here to download Figure Figure-6B.pdf

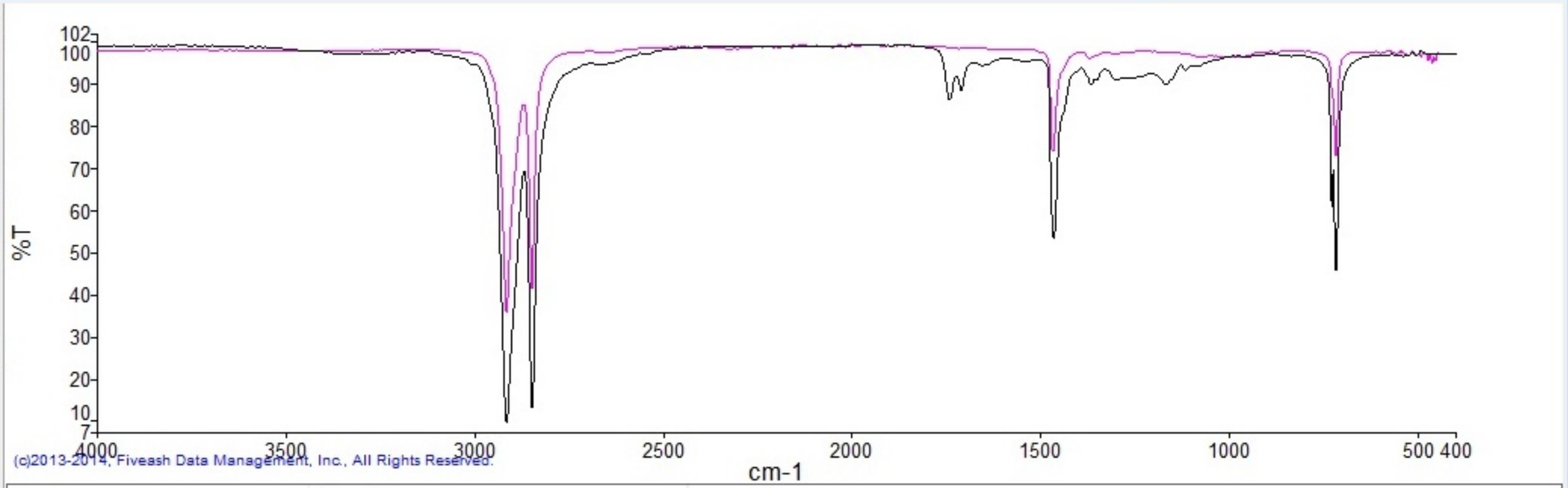
Search Results:

Sample Name	Search Score	Search Best Hit	Search Best Hit Description
essai2 cut	0,990034	P01034	P1034.SP IMPACT-RESISTANT UHMW POLYETHYLENE ROD

Search Hit List

	Search Score	Search Reference Spectrum Description
1	0,990034	P1034.SP IMPACT-RESISTANT UHMW POLYETHYLENE ROD
2	0,987849	P0508.SP POLYETHYLENE, MN=1100, 9002-88-4
3	0,985468	P0511.SP POLYETHYLENE, MN=6500, 9002-88-4
4	0,980933	P0509.SP POLYETHYLENE, MN=1400, 9002-88-4
5	0,976216	P1047.SP SELF LUBRICATING OIL-FILLED UHMW POLYETHYLENE ROD
6	0,976013	H192.SP POLYETHYLENE 617A, COSMETIC RAW MATERIAL
7	0,975453	P1036.SP METAL-DETECTOR-GRADE UHMW POLYETHYLENE ROD
8	0,975156	P0510.SP POLYETHYLENE, MN=1800, 9002-88-4
9	0,973899	P1037.SP MOISTURE-RESISTANT LDPE POLYETHYLENE ROD
10	0,970228	P0134.SP ETHYLENE/METHACRYLIC ACID COPOLYMER, METHACRYLIC ACID 4%, 2505

Accept as Best Hit | Search Residuals



Name	Description
essai2 cut	Sample 529 By mvirsek Date petek, septem...
P01034	P1034.SP IMPACT-RESISTANT UHMW POL...

Additional properties of P01034

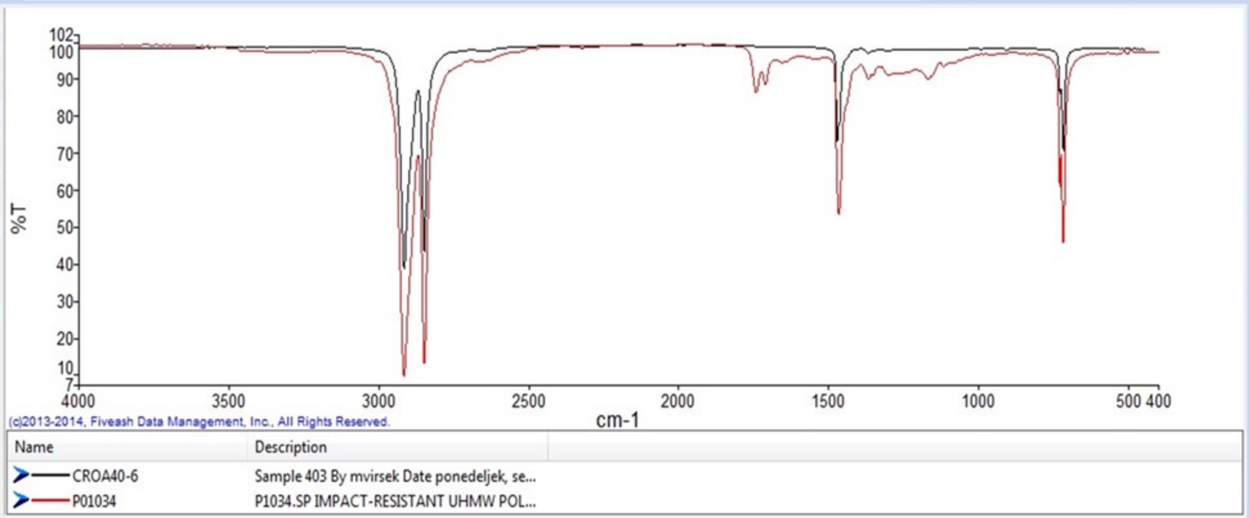
Property	Information
Copyright	(c)2013-2014, Fiveash Data Management, Inc., All Rights Reserved.

Figure 7

Click here to download Figure Figure-7A-D.pdf

7A

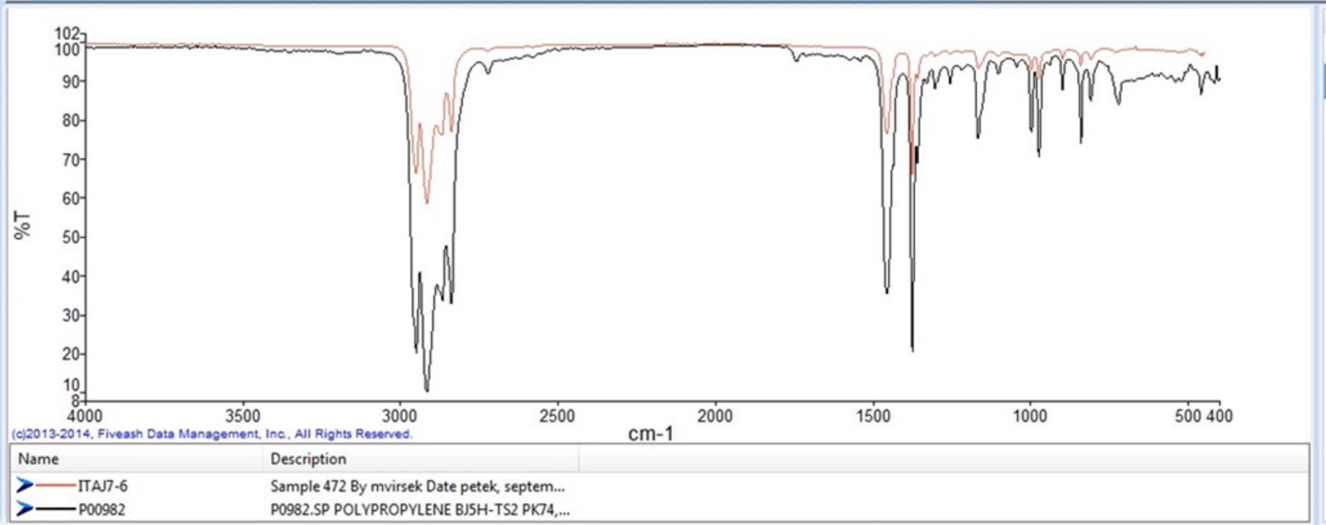
Sample Name	Search Score	Search Best Hit	Search Best Hit Description
CROA40-6	0,990008	P01034	P1034.SP IMPACT-RESISTANT UHMW POLYETHYLENE ROD



Name	Description
CROA40-6	Sample 403 By mvirsek Date ponedeljek, se...
P01034	P1034.SP IMPACT-RESISTANT UHMW POL...

7B

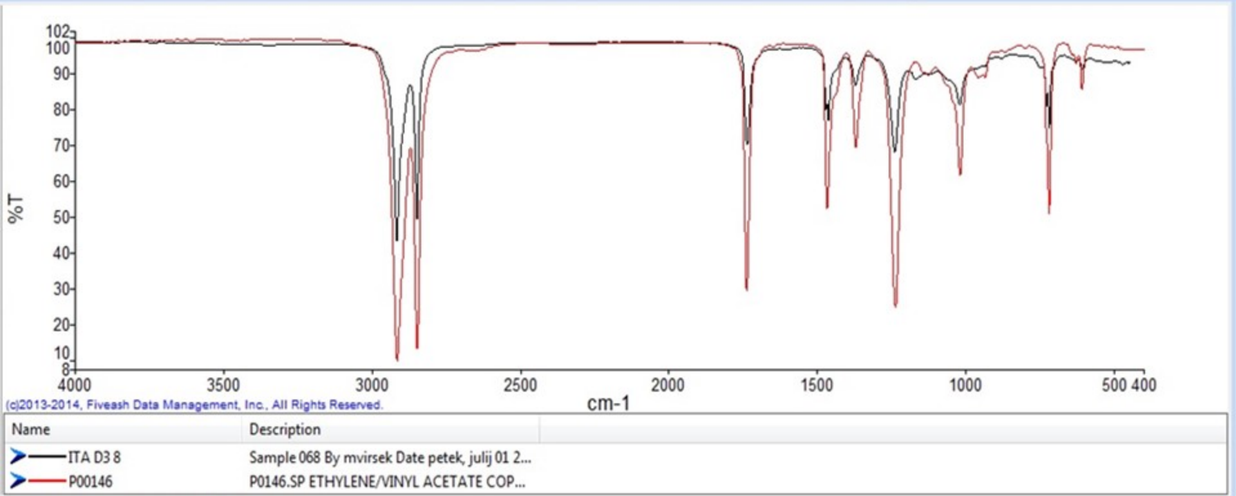
Sample Name	Search Score	Search Best Hit	Search Best Hit Description
ITAJ7-6	0,989211	P00982	P0982.SP POLYPROPYLENE BJ5H-TS2 PK74, DARK GRAY, ADVANCED C...



Name	Description
ITAJ7-6	Sample 472 By mvirsek Date petek, septem...
P00982	P0982.SP POLYPROPYLENE BJ5H-TS2 PK74,...

7C

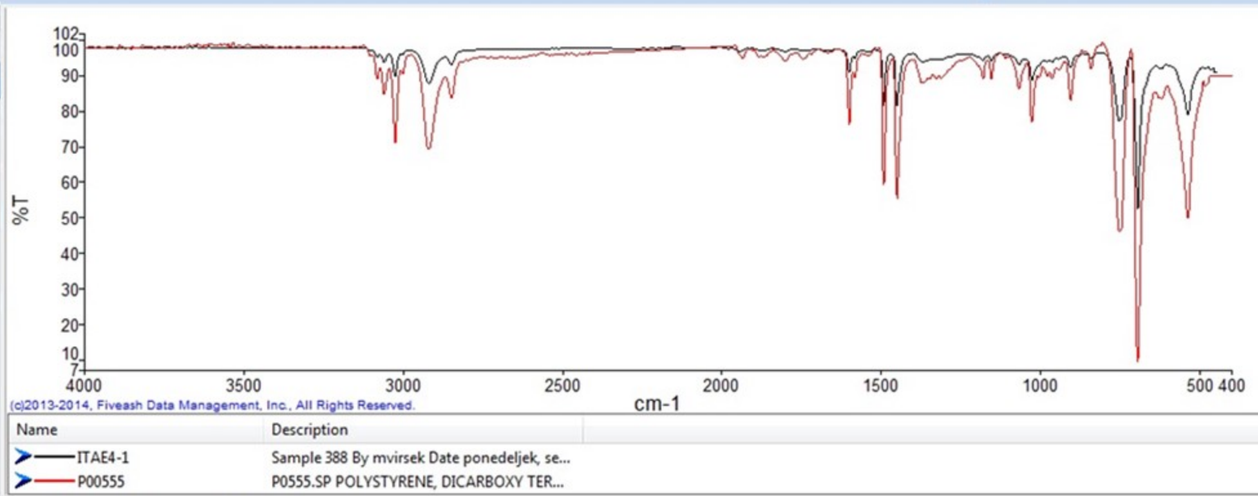
Sample Name	Search Score	Search Best Hit	Search Best Hit Description
ITA D3 8	0,970323	P00146	P0146.SP ETHYLENE/VINYL ACETATE COPOLYMER, 14% VA BY WT., 249...



Name	Description
ITA D3 8	Sample 068 By mvirsek Date petek, julij 01 2...
P00146	P0146.SP ETHYLENE/VINYL ACETATE COP...

7D

Sample Name	Search Score	Search Best Hit	Search Best Hit Description
ITAE4-1	0,995725	P00555	P0555.SP POLYSTYRENE, DICARBOXY TERMINATED, MW=150000, 9003-...



Name	Description
ITAE4-1	Sample 388 By mvirsek Date ponedeljek, se...
P00555	P0555.SP POLYSTYRENE, DICARBOXY TER...

Compounds	Tr (min)	Calibration curve equation	R^2	Recovery (%)
α-HCH	9.25	y = 1836x – 315	0.9992	99
γ-HCH	9.92	Y = 2055x – 158	0.9996	96
β-HCH	10.45	Y = 772x + 58	0.9993	78
24-DDE	13.90	y = 2611x + 262	0.9999	76
Endosulphan I	14.50	y = 2015x + 280	0.9999	74
44-DDE	15.16	y = 3942x – 427	0.9988	82
24-DDD	15.52	y = 1822x + 157	0.9999	94
24-DDT	16.64	y = 962x – 93	0.9965	75
44-DDD	17.11	y = 2617x + 44	0.9992	86
Endosulphan II	17.30	y = 2212x + 123	0.9995	102
44-DDT	18.32	y = 725x – 80	0.9955	96

Compounds	Tr (min)	Calibration cu	R2	Recovery (%)
a-HCH	9.25	y = 1836x – 3	0.9992	99
g-HCH	9.92	Y = 2055x – 1	0.9996	96
b-HCH	10.45	Y = 772x + 58	0.9993	78
24-DDE	13.90	y = 2611x + 2	0.9999	76
Endosulphan	14.50	y = 2015x + 2	0.9999	74
44-DDE	15.16	y = 3942x – 4	0.9988	82
24-DDD	15.52	y = 1822x + 1	0.9999	94
24-DDT	16.64	y = 962x – 93	0.9965	75
44-DDD	17.11	y = 2617x + 4	0.9992	86
Endosulphan	17.30	y = 2212x + 1	0.9995	102
44-DDT	18.32	y = 725x – 80	0.9955	96

Table 2 Tiff

Sample Name	Search Score	Search Best Hit	Search Best Hit Description
Sample 1-1	0.990764	P01034	P1034.SP IMPACT-RESISTANT UHMW POLYETHYLENE ROD
Sample 1-2	0.992768	P00508	P0508.SP POLYETHYLENE, MN=1100, 9002-88-4
Sample 1-3	0.990528	P01037	P1037.SP MOISTURE-RESISTANT LDPE POLYETHYLENE ROD
Sample 1-4	0.956303	P00561	P0561.SP POLYSTYRENE, MONOCARBOXY TERMINATED, MW=200000, 9003-53-6
Sample 1-5	0.988493	P00147	P0147.SP ETHYLENE/VINYL ACETATE COPOLYMER, 18% VA BY WT., 24937-78-8
Sample 1-6	0.990185	P01046	P1046.SP RIGID HDPE POLYETHYLENE ROD
Sample 1-7	0.988167	P01034	P1034.SP IMPACT-RESISTANT UHMW POLYETHYLENE ROD
Sample 1-8	0.969821	P00546	P0546.SP POLYPROPYLENE, ISOTACTIC, TG=-26, 9003-07-0
Sample 1-9	0.991779	P01036	P1036.SP METAL-DETECTOR-GRADE UHMW POLYETHYLENE ROD
Sample 1-10	0.988388	P01046	P1046.SP RIGID HDPE POLYETHYLENE ROD

Sample Name	Search Score	Search Best Hit
Sample 1-1	0.990764	P01034
Sample 1-2	0.992768	P00508
Sample 1-3	0.990528	P01037
Sample 1-4	0.956303	P00561
Sample 1-5	0.988493	P00147
Sample 1-6	0.990185	P01046
Sample 1-7	0.988167	P01034
Sample 1-8	0.969821	P00546
Sample 1-9	0.991779	P01036
Sample 1-10	0.988388	P01046

Search Best Hit Description
P1034.SP IMPACT-RESISTANT UHMW POLYETHYLENE ROD
P0508.SP POLYETHYLENE, MN=1100, 9002-88-4
P1037.SP MOISTURE-RESISTANT LDPE POLYETHYLENE ROD
P0561.SP POLYSTYRENE, MONOCARBOXY TERMINATED, MW=200000, 9003-53-6
P0147.SP ETHYLENE/VINYL ACETATE COPOLYMER, 18% VA BY WT., 24937-78-8
P1046.SP RIGID HDPE POLYETHYLENE ROD
P1034.SP IMPACT-RESISTANT UHMW POLYETHYLENE ROD
P0546.SP POLYPROPYLENE, ISOTACTIC, TG=-26, 9003-07-0
P1036.SP METAL-DETECTOR-GRADE UHMW POLYETHYLENE ROD
P1046.SP RIGID HDPE POLYETHYLENE ROD

Excel Spreadsheet- Table of Materials/Equipment

Click here to download Table of Materials/Equipment Materials List.xlsx

Name of Material/ Equipment	Company	Catalog Number
Alpha–HCH	Dr. Ehrenstorfer, Augsburg, Germany	DRE-C14071000
Beta–HCH	Fluka, Sigma-Aldrich, St. Louis, USA	33376-100MG
Lindane	Fluka, Sigma-Aldrich, St. Louis, USA	45548-250MG
Endosufan I	Supleco, Sigma-Aldrich Bellefonte, PA, USA	48576-25MG
Endosulfan II	Supleco, Sigma-Aldrich, Bellefonte, PA, USA	48578-25MG
2,4'–DDD	Fluka, Sigma-Aldrich, St. Louis, USA	35485-250MG
4,4’–DDD	Dr. Ehrenstorfer, Augsburg, Germany	DRE-C12031000
2,4’–DDE	Dr. Ehrenstorfer, Augsburg, Germany	DRE-C12040000
4,4’-DDE	Fluka , Sigma-Aldrich, St. Louis, USA	35487-250MG
2,4’–DDT	Dr. Ehrenstorfer, Augsburg, Germany	DRE-C12081000
4,4’–DDT	National Institute of Standards and Technology, (	RM8469-4,4'-DDT
n-Hexane	VWR International GmbH, Graumanngasse, Viena, Austria	83992.320
Acetone for HPLC	J.T.Baker, Avantor performance Materials B.V., Teugseweg, Netherlands	8142
FL-PR Florisil 1000mg/6mL	Phenomenex, Torrance, CA, USA	8B-S013-JCH
Fat free quartz sand 0.3-0.9 mm	Buchi, Flawil, Switzerland	37689
Gas chromatograph Hawlett Packard HP 6890 Series gas chromatograph with GERSTEL MultiPurpose Sampler MPS 2XL with ECD and FID detector	Agilent technologies, Santa Clara USA	
Presure fluid extractor, Speed Extractor E-916	Buchi, Flawil, Switzerland	
Solid phase extractor	Supleco, Sigma-Aldrich Bellefonte, PA, USA	
Concentrator miVac DUO	Genevac SP Scientific, Suffolk UK	
GC capillary column Zebron ZB-XLB (30 x 0.25 x 0.25)	Phenomenex, Torrance, CA, USA	122-1232
ATR FT-IR Spectrometer, Spectrum-Two	Perkin Elmer	

Comments/Description
H301, H351, H400, H410, H312
H301, H312, H351, H410
H301, H312, H332, H362, H410
H301, H410
H301, H410
H351
H301, H351, H400, H410, H312
H351, H400, H410, H302
H302, H351, H410
H301, H311, H330, H351, H400, H410
H301, H311, H351, H372, H410
H225, H315, H336, H373, H304, H411
H225, H319, H 336

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: EXTRACTION OF ORGANOCHLORINE PESTICIDES ADSORBED ON PLASTIC PELLETS USING A PRESSURIZED FLUID EXTRACTOR AND PLASTIC TYPE DETERMINATION

Author(s): M. PFLIEGER, P. MAKORIČ, M. KOVAČ VIRŠEK, Š. KOREN

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via: [X] Standard Access [ ] Open Access

Item 2 (check one box):

[X] The Author is NOT a United States government employee.

[ ] The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

[ ] The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

ARTICLE AND VIDEO LICENSE AGREEMENT

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name:	MARILYNE PFLIEGER
Department:	LABORATORY FOR ENVIRONMENTAL RESEARCH
Institution:	UNIVERSITY OF NOVA GORICA
Article Title:	EXTRACTION OF ORGANOCHLORINE PESTICIDES ADSORBED ON PLASTIC PELLETS USING A PRESSURIZED FLUID EXTRACTOR AND PLASTIC TYPE DETERMINATION
Signature:	 Date: 27.9.2016

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

Reply to Reviewer #1

We thank the reviewer for his/her comments. We took them into account and we have changed our manuscript accordingly.

Manuscript Summary:

The idea of a visual paper on new or adapted methods is very useful for the scientific community. This paper was a nice reading and watching. methods are carefully explained, step by step, which permit the audience to repeat and even improve the alternative method used by authors to quantity pesticides adsorbed on microplastics and identify polymer types.

Few comments on the paper:

-Figures 4A and B, 6A, 6B, 7 A-D, are in very poor quality. Please replace for better figures.

The mentioned figures have been replaced by files of better quality. The figures 7A, 7B, 7C and 7D have been merged as one figure.

-Reference 7 is a very cited reference but it is out-of-date. I suggest the authors replace with a newer one.

The reference 7 of Derraik et al. 2002 has been replaced by the one of "Cole et al. 2011":

Cole, M.; Lindeque, P.; Halsband, C.; Galloway, T.S. Microplastics as contaminants in the marine environment: A review. *Mar.Pollut. Bull.* **62**(12), 2588–2597, DOI: 10.1016/j.marpolbul.2011.09.025 (2011).

Major Concerns:
N/A

Minor Concerns:
N/A

Additional Comments to Authors:
N/A

Reply to Reviewer #2

We thank the reviewer for his/her comments. We took them into account and we have changed our manuscript accordingly.

Manuscript Summary:
I would like to congratulate the authors for this step-by-step guide to the extraction of pesticides adsorbed to plastic pellets. The video that complements the protocol is self-explanatory and provides valuable information in some of the steps, regarding either health and safety regulations or ways to protect the sample from airborne contamination.

Major Concerns:
None to be noted.

Minor Concerns:
Probably figure 7 could somehow be improved with a composite of all figures.

The figures 7A, 7B, 7C and 7D have been merged as one figure as suggested by the reviewer.

Additional Comments to Authors:
I understand the concern reflected on lines 511-512, on finding alternative separation techniques that preserve the chemicals, as these techniques might influence the results of adsorbed pollutants. What kind of alternative would you suggest?

This issue is quite challenging and up to now the safest method in this regard remains the visual sorting of microplastics from other material, directly or under stereomicroscope. However, the limitation lies in the fact that visual inspection and identification is only possible for large pieces (>0.5-1 mm). Below this size, other types of organic or inorganic fragments can be easily mistaken for plastic particles therefore the analysis of the sorted sample is required to assess its chemical nature (e.g. by FT-IR). The purification of plastic sample is obligatory prior to this analysis to avoid artifacts due to the presence of impurities (e.g. biofilms). This purification step, which usually involves the use of strong oxidants/acids and/or organic solvents eventually under heat and sonication, will lead to the desorption and/or degradation of the adsorbed contaminants. Other purification methods, which might be less aggressive towards the adsorbed pollutants, have been tested with success such as the use of sequential enzymatic digestion (Cole and al., 2014).

Thus, for larger pieces, the visual inspection should be applied when the aim is the determination of adsorbed pollutants, as well as microorganism. For smaller pieces, for the determination of which the chemical analysis is required, the use of an analytical technique that is not strongly affected by the presence of impurities should be selected since all type of purification steps will impact the results of adsorbed contaminants to some extent. Some recent studies have investigated the possibility of using pyrolysis-GC in combination with mass spectrometry to identify the plastic type (Fries et al., 2013). This technique might be less sensitive to impurities since the analysis is based on the thermal degradation products and their separation. Finally, if a purification step is required the enzymatic digestion method might be the most appropriate.

Cole, M.; Webb, H.; Lindeque, P. K.; Fileman, E. S.; Halsband, C.; Galloway, T. S. Isolation of microplastics in biota-rich seawater samples and marine organisms. Scientific Reports, 4, 4528, (2014).

Fries, E.; Dekiff, J.H.; Willmeyer, J.; Nuelle, M.T.; Ebertc., M.; Remy., D. Identification of polymer types and additives in marine microplastic particles using pyrolysis-GC/MS and scanning electron microscopy. Environ. Sci.: Processes Impacts, 15, 1949-1956, (2013).

Changes recommended by the JoVE Scientific Review Editor

We thank the scientific review editor for his/her valuable comments. We took them into account and we have changed our manuscript and the video accordingly.

The manuscript has been modified by the Review Editor to comply with the JoVE formatting standard. Please maintain the current formatting throughout the manuscript. The updated manuscript (55531_R0_121316) is in your Editorial Manager account. In the revised PDF submission, there is a hyperlink for downloading the .docx file. Please download the .docx file and use this updated version for any future revisions. The updated manuscript is also attached.

• General Formatting:
1) Please thoroughly review the language and grammar prior to resubmission. Please copy-edit the entire manuscript for any grammatical errors you may find. The text should be in American-English only. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.

The manuscript was edited for grammatical errors and for American-English spelling.

2) JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Please remove all commercial sounding language from your manuscript (text and figures). All commercial products should be sufficiently referenced in the table of materials/reagents. Here are some examples from your manuscript – Florisil.
Florisil has been replaced by “activated magnesium silicate sorbent”,

3) If you are re-using figures from a previous publication, please obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."
All figures are original and none of them are re-used from a previous publication.

• Abstract:
1) Line 47, short abstract - No abbreviations please – ATR FT-IR
The abbreviation has been replaced by full name.

2) Line 55, long abstract - No abbreviations please – PCBs, DDT, DDE, DDD
The abbreviations PCBs and DDT have been replaced by full names. DDE and DDD have been deleted.

• Introduction:

1) Line 122 - - Please define all abbreviations at first use - POPs

The abbreviations have been defined.

• Protocol:

1) Please re-write General considerations, Line 151 to 162 as step 1 in imperative tense, as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.) This will help the reader to prepare sampling materials ahead of time and will bring a continuity in the protocol.

This paragraph has been rewritten according to the comment and inserted as different steps of the protocol.

2) Line 142 and rest of the protocol – Please use a single space between the numerical value and unit. For example, 25 degC.

This has been corrected throughout the manuscript.

3) Line 166, 170 - The text should be in American-English only. Replace words such as "colour" by "color".

"Colour" has been replaced by "color".

4) Please write the General considerations, Line 209 to 226 as a separate step, similar to the first comment above.

This paragraph has been rewritten according to the comment and inserted as different steps of the protocol.

5) Line 297 – please write the appropriate step, section number in the place of 2.3 after adding the steps mentioned above.

The number of the step has been corrected according to the above corrections.

6) Line 322 – replace "specify" by "specificity"

"Specify" has been replaced by "specificity".

• Figure legends:

1) Please provide a clear title for figure legends on line 339.

The legend has been changed.

2) Line 344, 345, figure legend 1B – please replace "rows" by "columns". Rows are horizontal vs. columns are vertical.

"Rows" has been replaced by "columns".

Comments on the Video -

Editing issues

• 1:56, 2:14, 2:18, 3:18, 4:02, 7:22 - During the fades here, a different clip is briefly visible. This looks like a mistake and should be fixed.

This issue has been fixed.

• 10:04-10:36 - The end of the video is about 30 seconds of black. This should be removed.
The black ending of 30 s has been removed.

Text/formatting issues
• 2:53, 4:50, 5:51 - The on screen text here is a bit difficult to read. I would suggest using a bolder font and adding a drop shadow to the text.
The changes have been made as suggested.

• 6:49-8:09, 9:29-9:34 - These two results discussions should be moved to their own chapter, which should be placed at the end of the protocol chapters. We understand why the authors placed each set of results after their respective processes, but for the purposes of navigation through the video it is beneficial to have a clearly-identified Results section for a viewer to access.
A new “Results” chapter has been included following the suggested indications.

• 9:35 - A numbered chapter title card reading "Conclusion" should be added here.
A new “Conclusion” card has been added.

Reply to Editorial comments

We thank the Editor for reviewing our manuscript JoVE 55531_R1. We addressed the different comments as shown here below.

Thank you very much for the revisions that were made to the manuscript in response to the Editorial comments. We appreciate your patience in working with us to ensure that the manuscript complies with JoVE's unique format and requirements. While majority of the Editorial comments have been addressed in your previous revision, the following comments need to be addressed in order for your manuscript to proceed to the next step of the review process. In the editorial manager, there is a hyperlink to download the .docx file. Please download the .docx file and use this updated version for future revisions.

1) Editor modified the formatting of the manuscript and adjusted spacing of the protocol section. Please read the entire manuscript carefully and make changes if required. Do not change the formatting.

We carefully read the manuscript and did not change the formatting.

2) Editor added track changes to the word document of your manuscript, attached to this email. Please approve/revise all track changes.

Done.

3) Please separate the figure legends from representative results. Make sure that the representative results refer to all the data figures.

We separated the figure and table legends from representative results. All figures and tables are cited in the text.

4) comment on the video – at 10.00 – need the title card showing manuscript title, author names [the same shown at the beginning of the video]. This card should be on the screen for at least 3 seconds.

The required titled card has been added in the video (5 s).

5) After you edit the video, in addition to uploading the low resolution video [less than 50 MB] to editorial manager, please upload a high resolution version without compression to the following link. All text in the video should be very legible, and there should be no pillar-boxing or letter-boxing. In general, most of the high-resolution video files received are several hundred megabytes in size. Please also include your manuscript number JoVE 55531 in the file name.
To upload the video file (up to 2 GB in size), please use the following link: http://www.jove.com/files_upload.php?src=16937003

Both videos have been prepared.