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## Sub- and supercritical fluid extraction of trichloropyridinol from soil prior to immunoassay

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### Abstract

A comparative study on the extraction of TCP (3,5,6-trichloro-2-pyridinol, a metabolite of chlorpyrifos) from soil with CO<sub>2</sub> and H<sub>2</sub>O is reported. The polarity of the analyte requires the presence of both a cosolvent (methanol) and an ion-pair reagent [(1*R*)-(-)-10-camphorsulfonic acid ammonium salt] for 95% extraction in 30 min when supercritical CO<sub>2</sub> at 40°C and 383 bar is used as extractant. Subcritical water (250°C and 200 bar) enables complete extraction within 15 min without additives. Quantitation of the target analyte is performed by specific immunoassay using a non-commercial monoclonal antibody which provides a linear determination range between 0.005 and 5 µg/g, with coefficients of variation of 5.3 and 4.9% for the SC-CO<sub>2</sub> and sub-H<sub>2</sub>O extractions, respectively. © 1997 Elsevier Science B.V.

**Keywords:** Trichloropyridinol; Water, sub-critical

### 1. Introduction

As a consequence of the widespread use of pesticides, the presence of their residues in the different environmental matrices has become an important issue in analytical science. There is a growing concern regarding the potential toxicity and/or ecotoxicity of the transformation products associated with these residues, which is demanding the development of appropriate analytical methods for their monitoring [1]. The application of immunoassay technology, as an alternative [2] or in conjunction with chromatographic techniques [3,4], is gaining acceptance for pesticide residue monitoring in the environment.

TCP (3,5,6-trichloro-2-pyridinol) is the major degradation product of chlorpyrifos [O,O-diethyl O-

(3,5,6-trichloro-2-pyridinyl) phosphorothioate] and chlorpyrifos-methyl [O,O-dimethyl O(3,5,6-trichloro-2-pyridinyl) phosphorothioate] insecticides and trichlopyr [(3,5,6-trichloro-2-pyridinyl)oxy]acetic acid] herbicide [5,6]. Both biotic and abiotic transformation processes yield TCP from parent pesticides in the environment, particularly in soil. TCP is a polar compound (see Fig. 1) that displays very different physical and chemical properties from the chlorpyrifos insecticides, and therefore exhibiting

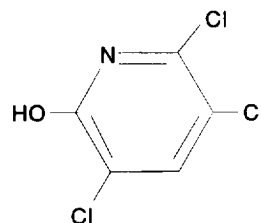


Fig. 1. Structure of TCP.

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different mobility and persistence in the environment [6].

One of the most significant contributions to solid sample extraction in the last few years has been supercritical fluid extraction (SFE) [7] or, better, supercritical fluid leaching. This technique emerged in the past decade as an excellent tool to overcome the difficulties of solid sample extraction. Despite the promising features of this technique, it has not fulfilled the expectations of researchers so far, maybe due to the non-ideal features of the supercritical fluids at present used as leaching agents. Thus, the main shortcoming of supercritical CO<sub>2</sub> is its low dielectric constant, which hinders the leaching of polar and ionic analytes or makes it difficult. The use of supercritical H<sub>2</sub>O yields very dilute solutions due to its liquid state under normal pressure and temperature; while other leaching agents such as ammonia, freons, organic solvents (ethanol, methanol, etc.) are not suitable due to their environmental aggressiveness.

The disadvantages of CO<sub>2</sub> as supercritical (SC) extractant can be minimized either by adding cosolvents, which increase the dielectric constant of the medium, or by decreasing the polarity of the target analytes [8]. The SFE of dioxins from sediments [9] is a good example of this assertion: virtually 100% of the analyte was extracted within 30 min from a sediment using SC-CO<sub>2</sub> containing 2% methanol; while, the standard Soxhlet extraction of the same sample for 18 h provided only 65% recovery. Another efficient way for improving SFE of polar analytes using CO<sub>2</sub> is ion-pair formation, as demonstrated in the extraction of clenbuterol from food samples [10].

Water is a leaching agent whose dielectric constant can be changed within a wide range by changing the temperature and pressure. The dilution effect can be circumvented by a concentration step after leaching, which can consist of either an in-line retention on a solid support packed in a column or in the flow-cell of a non-destructive detector, the latter enabling monitoring of the kinetics of the extraction process [11], or a liquid–liquid extraction, evaporation, etc. Thus, the determination of organic pollutants from environmental solids has been developed using water to extract polar, moderately polar and non-polar organics increasing the extraction temperature from

50°C (subcritical water) to 400°C (supercritical water). Although polar organics are easily extractable with water, quantitative extraction of low-polarity organics such as polyaromatic hydrocarbons (PAHs) can be achieved at 50 bar and 250°C with significantly higher recoveries (89–112%) than those reported by NIST based on 48 h Soxhlet extractions [12]. Biochemical use of SC-H<sub>2</sub>O extraction of the enzymic hydrolysis of testosterone-β-D-glucuronide has recently been reported [13]. Water extraction under subcritical conditions which is also called high-pressure solvent extraction [14] and accelerated solvent extraction [15] is commonplace at present. An additional use of subcritical water is as eluent for reversed-phase HPLC [16]. A recent comparison of extraction techniques such as supercritical fluid, Soxhlet and high-pressure solvent extraction (HPSE) for leaching of organophosphorus compounds from soil shows that HPSE provides extraction efficiencies comparable with those obtained with Soxhlet and SFE but with substantial saving of time and cost [17].

In the present research a comparative study on the use of CO<sub>2</sub> and H<sub>2</sub>O as extractants of TCP has been developed, showing the advantages and disadvantages involved in the use of each.

## 2. Experimental

### 2.1. Instruments and apparatus

A Hewlett–Packard 7680A supercritical fluid extractor equipped with a 7-ml thimble as extraction cell and a packing of small stainless-steel balls as analyte trap was used to perform the SC-CO<sub>2</sub> extractions.

The subcritical water extractions were carried out using the following assembly: A Knauer 64 HPLC pump with digital flow-rate and pressure readouts was used to impel the extractant. An extractor (a prototype designed and patented by Salvador and Merchán, [18]) consisting of a stainless steel cylindrical extraction chamber, (8 cm×3 mm I.D.), and closed with screws at either end that permit the circulation of the leaching fluid through them was used. The screw caps also contain stainless steel filter plates (2 μm in thickness and 1/4 inch I.D.) to

ensure that the solid material remains in the extraction chamber. This chamber, together with a stainless steel preheater is located in an oven, designed to work up to 300°C and controlled using a Toho TC-22 temperature controller. A cooler system (consisting of a coil coupled to an Ultraterm 6000383 P-Selecta recirculation bath) was used to cool the fluid from the oven to a constant temperature close to 25°C. The outlet of this coil was coupled to a home-made variable restrictor in order to control the pressure in the leaching chamber.

Flat-bottomed polystyrene ELISA plates (Costar High Binding 3590, Cambridge, MA, USA) and an ELISA plate reader (Mios, Merck) were used for development of the immunoassay step.

## 2.2. Materials

Stock standard solution of 400  $\mu\text{M}$  TCP (generously donated by DowElanco) in *N,N'*-dimethylformamide (Panreac, Spain) was prepared. The synthesis of the haptene 1, preparation of the HRP (horse-radish peroxidase)-1 enzyme tracer and production of the monoclonal antibody (LIB-MC2) have been described elsewhere [19].

SFE/SFC-grade  $\text{CO}_2$  (Air Products) and bidistilled degassed water purified through a Milli-Q deionizing unit (Millipore) were used as extractants.

Ion-pair reagent solution of (1*R*)-(-)-10-camphorsulfonic acid ammonium salt (Aldrich) 0.1 *M* in HPLC-grade methanol (Romil Chemicals) was used to enhance the recovery of the SC- $\text{CO}_2$  extractions. HPLC-grade methanol (Romil Chemicals) was also used with this purpose.

Coating buffer containing 50 *mM* carbonate–bicarbonate, pH 5.4 (Merck, Darmstadt, Germany); washing aqueous solution of 0.15 *M* NaCl (Merck) containing 0.05% Tween 20 (Sigma). PBS was the assay buffer, which contains 10 *mM* phosphate, 137 *mM* NaCl and 2.7 *mM* KCl (all from Merck), pH 7.5 and PBST is the PBS buffer containing also 0.05% Tween 20 (Sigma). Substrate solution of *o*-phenylenediamine (Merck) of 2 mg/ml containing 0.012%  $\text{H}_2\text{O}_2$  (Panreac, Spain) in 25 *mM* citrate (Merck), 62 *mM* sodium phosphate (Merck) buffer (pH 5.4) and 2.5 *M*  $\text{H}_2\text{SO}_4$  (Panreac) were used in the immunoassay step.

## 2.3. Sample preparation

A weighed portion of calcareous soil (0.5 g for  $\text{CO}_2$  extractions and 0.1 g for water extractions) was spiked with standard solution of TCP of 0.005, 0.05, 1 and 5  $\mu\text{g/g}$ . The addition of 0.5 ml of ion-pair reagent methanolic solution and 1 ml of pure methanol was required for the  $\text{CO}_2$  extractions. When water was used as leaching agent, an amount of  $\sim 0.04$  g of diatomaceous earth needed to be mixed with the sample in order to avoid plugging.

## 2.4. Procedure

### 2.4.1. SC- $\text{CO}_2$ extractions

Extractions were performed using the HP extractor. The  $\text{CO}_2$  was delivered from a cylinder supplied with a dip tube, aspirated by a double piston pump and passed through the 7-ml extraction cell which contained the sample. The leached TCP was driven to a 0.45-ml stainless steel bead trap through an automated variable-diameter restrictor which virtually avoided plugging and provided a constant flow-rate during the extraction process. In a subsequent step (rinsing), a PBS buffer stream at a flow-rate of 0.5 ml/min was pumped through the trap by a syringe pump. In order to ensure that no methanol was retained in the trap, the temperature of this device was 70°C during the extraction step. Restrictor and trap temperature was 60°C during the rinsing step. The extract was collected in weighed vials and the weight of the extract was calculated. In all cases, except for the lowest level of TCP (0.005  $\mu\text{g/g}$ ), PBS-buffer dilutions (1:10, 1:25 and 1:50 for 0.05, 1 and 5  $\mu\text{g/g}$ , respectively) prior to ELISA were required in order to obtain a suitable absorbance value. The recoveries were calculated from TCP concentrations determined by ELISA and the volume of extract+diluter; the latter being obtained from weight/density data, which were more precise than those from volume data. The study of the alternatives described in Section 3.1.1 was tested using the following working conditions:  $\text{CO}_2$  flow-rate, 1 ml/min; pressure, 281 bar ( $\text{CO}_2$  density, 0.9 g/ml); extraction chamber temperature, 40°C and extraction time, 15 min.

#### 2.4.2. Sub-critical H<sub>2</sub>O extractions

Extractions were performed using the assembly described above. Degassed Milli-Q water stored in a reservoir was pumped to the oven, where it reached the preheater and passed through the 1-ml extraction chamber, which contained the sample. The leached TCP was cooled in the refrigerant at 25°C. The pressure of the system was controlled by a manually actuated variable restrictor that was immersed in a weighed glass tube and the weight of the extract was calculated. As the volume of the extract was higher than that obtained from the CO<sub>2</sub> extractions, PBS-buffer dilutions of 1:2, 1:5 and 1:25 were required for concentration levels of 0.05, 1 and 5 µg/g, respectively in order to obtain a suitable absorbance and suitable working conditions in the ELISA step. The recoveries were calculated as described in the SC-CO<sub>2</sub> procedure.

#### 2.4.3. ELISA quantitation of TCP

ELISA plates were coated overnight at room temperature with 100 µl/well of 0.5 µg/ml of LIB-MC2 monoclonal antibody in coating carbonate–bicarbonate buffer. The plates were washed four times with the washing solution. Then, 50 µl of standard or sample were added to each well of coated-antibody plates, followed by 50 µl of 15 ng/ml of HRP-TS1 in assay PBST-buffer. The plates were incubated for 1 h at room temperature. After washing, the HRP activity bound to the wells was measured by adding 100 µl of the substrate solution. After 10 min, the reaction was stopped by adding 100 µl of 2.5 M sulphuric acid and the absorbance was read in the dual-wavelength mode (490–630 nm) using a plate reader. The signal thus obtained was used to quantify the leached TCP. The cali-

bration curve was a typical sigmoidal curve, fitted by means of the suitable software. The equation obtained by the iterative method was:

$$c = 0.0548138(0.905503 - A/A) - 0.0109551^{(1/1.39883)}, \quad (1)$$

where  $c$  and  $A$  denote concentration of TCP expressed in µg/l of extract and absorbance, respectively; and the parameter  $\chi^2$  (correlation coefficient of the sigmoidal curve) was 2.7755E-5.

### 3. Results and discussion

#### 3.1. Optimisation of variables

The experimental variables were optimised in both methods in order to maximize recovery of TCP as quickly as possible. The soil was spiked with 0.05 µg/g of TCP for this study. The univariate method was used in all instances. Common and characteristic variables of each method were studied and the optimum values for both methods are given in Table 1. The influence of the ELISA variables was studied in previous works [19].

##### 3.1.1. Characteristic variables of the extractions with SC-CO<sub>2</sub>

Preliminary experiments were performed to overcome poor recoveries (<16%) of TCP due to the polar nature of the analyte. With this aim two methods were tested: (a) addition of a modifier (methanol) to the sample, in order to increase the polarity of the extractant and (b) addition of an ion-pair reagent to the sample, in order to lower the

Table 1  
Optimisation of variables

Type	Variables	Optimum value	
		H <sub>2</sub> O ext.	CO <sub>2</sub> ext.
Common	Flow-rate (ml/min)	4	1
	Pressure (bar)	200	383
	Temperature (°C)	250	40
	Extraction time (min)	15	30
Characteristic	Cosolvent (methanol) (ml)		1.0
	0.1 M IPR <sup>a</sup> (ml)	–	0.5

<sup>a</sup> IPR, ion-pair reagent.

Table 2  
Effect of a cosolvent on the recovery of TCP from soil with SC-CO<sub>2</sub><sup>a</sup>

Additive	Recovery (%)
None	15.4
Methanol	57.4
Methanol + IPR <sup>b</sup>	80.0

<sup>a</sup> Working conditions are given in the text.

<sup>b</sup> IPR, ion-pair reagent.

polarity of TCP and thus increase its solubility in SC-CO<sub>2</sub>. The recoveries obtained for these preliminary studies are summarized in Table 2.

#### 3.1.1.1. Addition of methanol

The methanol was directly added to the sample in the chamber. The use of this cosolvent enhanced the extraction efficiency, providing a recovery of TCP from soil of 57% versus 15% obtained when no modifier was added.

#### 3.1.1.2. Addition of methanolic solution of the ion-pair reagent

The addition of *R*-10 camphorsulfonic acid ammonium salt to the chamber prior to extraction provided a recovery of TCP from soil of 80%, better than that obtained when methanol was added to the sample as cosolvent (57%) or when pure CO<sub>2</sub> was used as extractant. Thus, this alternative was selected as optimum for development of the SC-CO<sub>2</sub> method.

### 3.1.2. Common variables

The pressure, temperature of the extraction chamber, flow-rate of the extractant (H<sub>2</sub>O or CO<sub>2</sub>) and extraction time were optimised in order to maximize extraction.

#### 3.1.2.1. Temperature of the extraction chamber

The temperature is the key variable when subcritical water is used as extractant. Its influence was studied between 50 and 250°C at a constant flow-rate of 4 ml/min, and a pressure of ~200 bar. The recovery increased drastically with increased temperature, as can be seen in Table 3. Thus, the latter value was selected for further experiments. The pressure and density are interrelated variables of a supercritical fluid. The effect of temperature for the SC-CO<sub>2</sub> extractions was investigated at a constant

Table 3  
Effect of the temperature on the recovery of TCP from soil with SubC-H<sub>2</sub>O<sup>a</sup>

Temperature (°C)	Recovery (%)
50	63.0
100	70.6
150	88.5
250	95.2

<sup>a</sup> Working conditions are given in the text.

CO<sub>2</sub> density of 0.8 g/ml, which allowed a wider range of temperatures than 0.9 g/ml, because of pressure limitation of the equipment used. Increase in temperature resulted in slightly higher recoveries (~10% when the temperature rose from 40–80°C).

#### 3.1.2.2. Pressure

When water was used as extractant, the effect of pressure was tested by modifying manually the diameter of the variable restrictor and working with a constant extraction temperature of 250°C. Pressures of 80 and 200 bar were tested, obtaining similar recoveries (~95%) in all instances. So, a pressure of ~200 bar was selected for further experiments. For the SC-CO<sub>2</sub> method the pressure was modified by changing the CO<sub>2</sub> density, obtaining recoveries for 80°C/164 bar and 40°C/383 bar of 87.5 and 94.7%, respectively. A value of 383 bars (the maximum provided by the HP extractor) was selected as the optimum, as it provided the best recovery.

#### 3.1.2.3. Flow-rate

This variable had practically no influence when SC-CO<sub>2</sub> was used as extractant. The recoveries obtained by increasing the flow-rate up to 100% were similar. For the accelerated leaching method, this variable was investigated using a chamber extraction temperature of 250°C and a pressure of ~200 bar. An increase of 1 ml/min of flow-rate provided a recovery increase of 21%, obtaining quantitative recovery of TCP within 15 min for a water flow-rate of 4 ml/min and the optimal values of the above optimised parameters, so this value was selected as optimum.

#### 3.1.2.4. Extraction time

The use of subcritical water under the optimal working conditions described above provided quan-

titative recovery of TCP from soil in 15 min, so this time was selected as optimum. The influence of this variable in the CO<sub>2</sub> extractions was studied also under the optimal working conditions and at least an extraction time of 30 min was required to obtain a TCP recovery of ~95%.

### 3.2. Features of the methods

The features of both the determination step and the overall process (extraction + determination) were studied separately.

The determination method obeyed a sigmoidal curve (Eq. (1)) within 0.002 and 158.736 µg/l. The precision, studied with 11 standard solutions of 0.277 µg/l of TCP, yielded an R.S.D. of 2.64%.

Spiked samples were prepared by adding different amounts of TCP to portions of soil, in order to know the range within which the target analyte could be optimally extracted. Table 4 shows the concentrations added, mean recovery and R.S.D. ( $n=2$ ) for each method. The range of linearity of both methods was 5 ng/g–5 µg/g. The recoveries obtained were quite similar in both cases and ranged from 94.6–102.8%, (average 98.1%) and a mean R.S.D. of 4.4% for SC-CO<sub>2</sub> extractions and from 95.7–99.9%, (average 98.5%) and a mean R.S.D. of 2.1% for SubC-H<sub>2</sub>O extractions.

The reproducibility of the overall process (SC-CO<sub>2</sub> extraction + ELISA and water extraction + ELISA) was studied by using seven samples of calcareous soil spiked with TCP at the 0.05 µg/g

Table 4  
Recoveries obtained by SC-CO<sub>2</sub> and SubC-H<sub>2</sub>O methods

Method	Spiked conc. (µg/g)	Mean recovery <sup>a</sup> (%)	R.S.D. <sup>a</sup> (%)
SC-CO <sub>2</sub>	0.005	100.6	4.2
	0.05	94.7	5.3
	1	102.8	2.9
	5	94.6	5.2
SubC-H <sub>2</sub> O	0.005	95.7	0.8
	0.05	99.7	4.9
	1	99.9	0.6
	5	98.9	2.0

<sup>a</sup>  $n=2$ .

level. The average recoveries thus obtained were 94.7 and 99.7% and the precision, expressed as R.S.D., was 5.3 and 4.9%, respectively.

### 3.3. Comparison of the methods

Subcritical water extraction combined with ELISA method and SC-CO<sub>2</sub> extraction combined with ELISA method, both developed for the determination of TCP in soils are compared in terms of efficiency and precision, extraction time, extraction temperature, pressure, additives, environmental pollution, preconcentration effect and cost, emphasizing the main advantages and disadvantages of each. These aspects are summarized in Table 5.

#### 3.3.1. Efficiency and precision

The precision provided by both extraction methods

Table 5  
Comparison of the SC-CO<sub>2</sub> and SubC-H<sub>2</sub>O methods

Aspect	SC-CO <sub>2</sub> + ELISA	SubC-H <sub>2</sub> O + ELISA
Mean recovery (%) <sup>a</sup>	94.7	99.7
Precision (as R.S.D. (%)) <sup>a</sup>	5.3	4.9
Extraction time (min)	30	15
Extraction temp. (°C)	40	250
Extraction pressure (bar)	383	200
Additives	Methanol, IPR <sup>b</sup>	None
Environmental pollution	Slight	Nil
Preconcentration effect	Yes	No
Cost (\$)	10 <sup>c</sup>	1 <sup>d</sup>

<sup>a</sup>  $n=7$ , (TCP)=0.05 µg/g.

<sup>b</sup> IPR = ion-pair reagent.

<sup>c</sup> The cost involves pure CO<sub>2</sub> for SFE + cryogenic CO<sub>2</sub> + ion-pair reagent + methanol + electrical consumption.

<sup>d</sup> The cost involves only electrical consumption.

is good and quite similar (R.S.D. ~5%). Slightly better recoveries are obtained when water is used as leacher (see Table 3).

### 3.3.2. Extraction time

An extraction time of 15 min provides quantitative recovery of TCP (99.7%) when subcritical water is used, versus 30 min required to reach a recovery of 94.7% by the SC-CO<sub>2</sub>-ELISA method. So, the water method is faster than the SFE method.

### 3.3.3. Extraction temperature

Temperatures of 40 and 250°C are needed for SC-CO<sub>2</sub> and H<sub>2</sub>O extractions, respectively. This rules out the application of the H<sub>2</sub>O-ELISA method to thermolabile analytes.

### 3.3.4. Pressure

Water extractions are performed at a pressure of ~200 bar. The pressure during SC-CO<sub>2</sub> extractions is fixed at 383 bar.

### 3.3.5. Additives

The polar character of TCP makes mandatory the presence of two additives (a cosolvent such as methanol, added to the sample to increase the polarity of the extractant and an ion-pair reagent, which reacts with the target analyte and gives rise to a less polar solute). These two additives enhance efficiency of the extraction with SC-CO<sub>2</sub>. No additives are required to obtain quantitative extraction of TCP with water.

### 3.3.6. Environmental pollution

Despite the fact that both methods can be classified as clean methods (sub- and supercritical fluid extraction avoid the use of great volumes of organic solvents which are required by conventional techniques as Soxhlet), CO<sub>2</sub> and small amounts of methanol (the added cosolvent) are emitted when SC-CO<sub>2</sub> extractions are performed, versus the absolutely clean extraction provided by water.

### 3.3.7. Preconcentration effect

The small volume for collection of the leached analyte provided by the SC-CO<sub>2</sub> (~1.4 ml) versus that obtained when water was used as extractant (~10 ml) is one of the main advantages of the

CO<sub>2</sub>-ELISA method. This preconcentration effect avoids subsequent preconcentration steps (such as adsorption columns, evaporation, etc.) that can be required after water extraction (due to the analyte dilution) if the detection method is not sensitive enough.

### 3.3.8. Cost

The availability of water with the required purity in the laboratory for sub- and supercritical extraction makes it a cheap alternative to SC-CO<sub>2</sub>. In addition, the equipment used for water extractions is twice as cheap as that required for CO<sub>2</sub> extractions.

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