*Article*

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# On-line Solid-Phase Extraction-Liquid Chromatographic Method for the Determination of Carbofuran and 7-Phenolcarbofuran in Cervical-Uterine Epithelial Tissue

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**Abstract.** A method for the trace level determination of carbofuran and 7-phenolcarbofuran in cervical-uterine epithelial tissue samples is proposed. The finely chopped tissue is methanol extracted in an ultrasonic bath; the extract is evaporated, diluted with water and acidified to eliminate hydrophobic impurities by precipitation. Preconcentration and additional cleanup are carried out in a polymeric (PLRP-S) precolumn by loading the filtrated extract and successively washing with acetonitrile-water 5:95 (v/v) and 0.01 M aqueous ammonia. The precolumn is finally on-line eluted and analysed by reverse-phase HPLC with UV detection at 280 nm. Recoveries >90% and relative standard deviation of ~12% were obtained in the analysis of non-contaminated tissue samples spiked at 0.2 μg/g of the pesticide and metabolite. Absolute limits of detection were 20 ng of analyte in a cervical biopsy (150-950 mg of epithelial tissue). Application to the analysis of three samples from countrywomen exposed to carbofuran revealed the probable presence of 7-phenolcarbofuran in two of them.
**Keywords:** Carbofuran, 7-phenolcarbofuran, trace analysis, cervical-uterine tissue.

**Resumen.** Se propone un método para determinación de carbofurano y 7-fenolcarbofurano a niveles traza en muestras de tejido epitelial cérvico-uterino. El tejido finamente dividido se extrae con metanol por ultrasonido; el extracto se evapora, diluye con agua y acidifica para eliminar impurezas hidrofóbicas por precipitación. La preconcentración y purificación adicional se realizan en una precolumna polimérica (PLRP-S) mediante la carga del extracto filtrado y lavados sucesivos con acetonitrilo-agua 5:95 (v/v) y solución acuosa de amoniaco 0.01 M. Finalmente, la precolumna se eluye en línea y analiza por cromatografía de líquidos en fase reversa con detección UV a 280 nm. Las recuperaciones obtenidas en el análisis de muestras de tejido no contaminado fortificadas con el pesticida y el metabolito a 0.2 μg/g fueron >90% con desviación estándar relativa de ~12%. Los límites absolutos de detección fueron de 20 ng de analito en una biopsia cervical (150-950 mg de tejido epitelial). La aplicación del método en el análisis de tres muestras, provenientes de mujeres campesinas expuestas al carbofurano, reveló la probable presencia de 7-fenolcarbofurano en dos de ellas.
**Palabras clave:** carbofurano, 7-fenolcarbofurano, análisis de trazas, tejido cérvico-uterino

## Introduction

Carbofuran (2,3-dihydro-2,2-dimethyl-7-bezofuranyl methylcarbamate) is a systemic *N*-methylcarbamate widely used in Mexico for the control of insects, nematodes and arachnida on a variety of agricultural crops including maize, alfalfa, sugar cane, watermelon, strawberry, banana and citrus. Due to its potent cholinesterase inhibition effect, carbofuran is also highly toxic to humans and wildlife (oral $LD_{50}$ in rats = 8 mg/Kg). This carbamate can penetrate into exposed organisms by inhalation, accidental ingestion or absorption through skin. Laboratory studies in rats have shown that the first symptoms of poisoning occur 15-30 min after lethal oral dose administration, accompanied by nausea, vomiting, increased mucous secretion in the lungs and airway obstruction which combined with pulmonary oedema finally provoke death [1]. In general, *N*-methylcarbamates have a relatively low persistence in the environment and do not present bioaccumulation; they are slowly hydrolysed in water and soil forming phenolic derivatives. Biotransformation of carbofuran in particular occurs by two main routes: via oxidation of carbon 3 to produce the derivatives 3-hydroxycarbofuran and 3-ketocarbofuran, or by hydrolysis of the carbamate ester producing the metabolites 7-phenolcarbofuran, 3-hydroxy-7-phenolcarbofuran and 3-keto-7-phenolcarbofuran. Studies in rats treated with small oral doses of carbofuran corroborated the presence of the derivatives, 3-hydroxy, 3-keto and 7-phenol in urine, whereas, the main metabolite in the urine of humans exposed to the pesticide was 7-phenolcarbofuran [1-3].

In recent years, an increasing number of countrywomen from agricultural regions where carbofuran is widely expanded have developed a cervical-uterine cancer. Although carbofuran is not considered a carcinogenic agent, the continuous contact of these women with the pesticide could have promoted its penetration and deposition on the extremely receptive cervical tissue provoking alterations that favoured the development of cancer. Therefore, it becomes of prime importance to develop a convenient method for the detection of carbofuran and/or its degradation products in cervical biopsies in order to verify this hypothesis.

Numerous analytical methods have been proposed for the trace level determination of *N*-methylcarbamates in surface water and groundwater [4-7], as well as in biological samples (fruits, vegetables, food) [7-14]; however only a few of them deal with the simultaneous determination of some metabolites in these complex matrices [15, 16]. The preferred analytical technique has been high performance liquid chromatography (HPLC) because most carbamates are thermally labile and not directly amenable to gas chromatography [5]. HPLC is often used in combination with post-column derivatization and fluorescence detection (i.e. EPA method 531.1) to achieve a high sensitivity and selectivity in the analysis of environmental samples [4, 5, 7, 8, 11-13]. The two-step post-column reaction consists on the hydrolysis of *N*-methylcarbamates to form methylamine that is sequentially derivatised in the presence of o-phtalic dicarboxaldehyde (OPA) and mercaptoethanol giving a highly fluorescent product [4, 12]. However, this procedure requires specialized and expensive instrumentation and, when applied to biological samples, a thorough cleanup of the extract is necessary prior to HPLC because many matrix components can interfere in the analysis [14]. Besides, this method cannot be used for the simultaneous determination of the phenolic carbamate metabolites because they do not react with OPA.

Considering the previous discussion, the aim of this work was to develop a method for the determination of trace amounts of carbofuran and 7-phenolcarbofuran in cervical-uterine epithelial tissue samples. HPLC with UV detection was used because both compounds give a convenient UV response, and also because this conventional instrumentation is commonly found in most analytical laboratories. The latter is important in view of the possible application of the method for the screening of samples from countrywomen exposed to the pesticide. However, it was necessary to design an on-line system, coupling sample preparation with HPLC analysis, to achieve the required sensitivity.

## Experimental

**Reagents**. HPLC-grade methanol (MeOH) and acetonitrile (AcCN) were from EM Science and Prolabo, respectively. Reagent water was obtained from a Nanopure deionizer (Barnstead Thermolyne, Model 04747). Other common reagents were analytical grade from Baker. Carbofuran and 7-phenolcarbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranol) were purchased from Chem Service. All reagents were used without further purification. Stock solutions of the pesticide and metabolite were prepared in AcCN (1000 mg/L) and stored in amber glass bottles at -20°C. Working standards of various concentrations were prepared by diluting appropriate aliquots of the stock solutions in MeOH. These standards were kept in amber glass bottles at 4°C when not in use and were renewed every 3-4 days.

**Instrumentation**. The liquid chromatograph was from Lab Alliance. It consisted of two Series III digital pumps, a Model 500 variable wavelength UV detector, a static mixer and an injection valve with a 27 μl loop (calibrated in the laboratory). Pump control and data acquisition and processing were performed by means of a Data Ally interface and the corresponding software installed in a Pentium III PC. A 7000 Rheodyne switching valve was inserted between the injector and the analytical column for the on-line coupling of sample preparation with HPLC analysis. The experimental set-up also consisted of an isocratic auxiliary pump (Eldex, Model CC-100-S) and a small stainless steel column (solid-phase extraction precolumn), both connected to the switching valve (Fig. 1). During

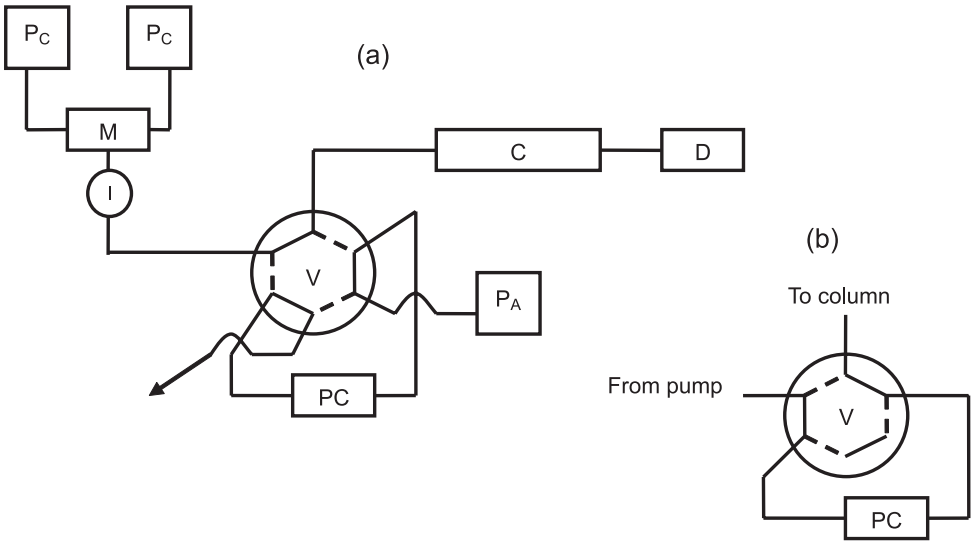


**Fig. 1**. Diagram of the experimental setup. $P_C$: chromatographic pumps, $P_A$: auxiliary pump, M: static mixer, I: injector, V: switching valve, PC: precolumn, C: analytical column, D: UV detector. Connected ports in the switching valve are indicated by solid lines. (a) Position of the valve during preconcentration and cleanup of the sample extract, (b) position of the valve during the on-line elution of the precolumn.

sample preparation, the extract obtained from epithelial tissue was loaded and purified in the precolumn by means of the auxiliary pump, while the analytical column was simultaneously equilibrated with the mobile phase sent by the HPLC pumps (Fig. 1a). Then, the valve was switched and the mobile phase flew through the precolumn eluting the trapped sample components and sending them to the analytical column for separation and analysis (Fig. 1b).

**Columns and Separation Conditions**. The reversed phase analytical column (150 × 4.6 mm i.d.) packed with Hypersil ODS 5 μm was obtained from Thermo Hypersil-Keystone. The solid-phase extraction (SPE) precolumn (20 x 2 mm i.d.) was home-packed with a polymeric styrene-divinylbenzene copolymer (10-15 μm PLRP-S from Polymer Laboratories) using a column packing system (Haskel, Model 29426). Different AcCN-water or MeOH-water mixtures with various additives (acid, buffers) were tested to obtain a good resolution of the analytes and their separation from the matrix interferences. The best results were obtained under isocratic conditions using an AcCN-$HClO_4$ (0.05 M) 18:82 (v/v) mobile phase. All separations were carried out at ambient temperature and at a flowrate of 1 mL/min. A detection wavelength of 280 nm was chosen for analysis.

**Sample Preparation**. Cervical biopsies from women that had not been exposed to carbofuran were used as blank samples for the development of the analytical method. Three other samples coming from exposed women were used for the application of the finally established method. All samples were carefully maintained at -20°C until use. The blank samples were thoroughly chopped and mixed to make an homogeneous lot from which small portions were taken for each assay. Some samples from this lot were fortified with known amounts of carbofuran and 7-phenolcarbofuran (in the range 20-100 ng) and left at rest for 12 hours to ensure the complete penetration and incorporation of the compounds to the tissue matrix. The cervical biopsies from exposed women were weighted and also thoroughly chopped prior to analysis.

Numerous experiments were carried out with the fortified and non-fortified blank samples to optimise the extraction, preconcentration and cleanup steps. The following procedure was finally adopted:

*Extraction*.- The chopped tissue is extracted with 5 mL of MeOH in an ultrasonic bath (Sonicor, Model SC-100) for 45 min. After separating the liquid, the solid residue is washed with 3 × 2-mL aliquots of fresh MeOH. The extract and washings are mixed and evaporated under a gentle $N_2$ stream to approximately 1 mL. Then, a 250 μL aliquot of concentrated formic acid and 1 mL of water are added, and the mixture is filtrated through a Nylon 66 membrane (47 mm, 0.45 μm, from LIDA) under vacuum (Millipore filtration unit, Model OM027). The membrane and retained precipitate are further rinsed with 1 mL of MeOH and 10 mL of water. All the liquid portions are collected in the same flask and made up to an approximate volume of 30 mL with water.

*Preconcentration and cleanup*.- The diluted extract is percolated through the reversed phase SPE precolumn mounted on the switching valve of the on-line set-up. Then, the precolumn is successively washed with 5 mL of AcCN-water 5:95 (v/v), 2 mL of a 0.01 M $NH_4OH$ solution adjusted to pH 11 with NaOH and again 5 mL of the AcCN-water mixture. After this treatment, the precolumn is ready for the on-line elution and HPLC analysis of the purified sample.

## Results and Discussion

The determination of polar pollutants at trace levels in biological samples is a challenging task because many compounds of similar polarity are present in these matrices at much higher concentration. In the present case, the problem is aggravated by the nature of the sample (a small and unique cervical-uterine biopsy) that do not offers the possibility of replicate analysis to adjust conditions or verify results; besides, the preconcentration of large sample amounts to increase sensitivity is excluded.

In order to simultaneously determine carbofuran and 7-phenolcarbofuran in the same sample, HPLC with UV detection was used. However, the moderate sensitivity and low selectivity of the detection system had to be compensated with a more elaborated sample pretreatment. Loading the whole sample extract in a precolumn, which was further on-line analysed, maximized the sensitivity of the method. Besides, all steps in the sample pretreatment process were carefully designed to reduce as much as possible the coextracted matrix interferences without losing the compounds of interest. The next sections describe the results obtained upon optimisation of conditions at each stage of the analytical method and give a rationale for the finally established procedure presented in the experimental part.

**HPLC Conditions**. Carbofuran and 7-phenolcarbofuran could be separated in reversed phase C-18 columns using acetonitrile-water or methanol-water mobile phases. Baseline separation of the two compounds required a relatively high retention (capacity factors >10), which was achieved by adjustment of the organic solvent content in the mobile phase. Coupling of the small precolumn (packed with the polymeric reversed phase PLRP-S) to the analytical column provoked an increase of retention and a strong widening and deformation of solute peaks when methanol-water mobile phases were used. The problem was notably reduced using acetonitrile instead of methanol; besides, the peak shape was additionally improved by acidification of the mobile phase. With the finally adopted mobile phase (AcCN-$HClO_4$ 0.05M, 18:82 v/v), retention times were 35-37 min for 7-phenolcarbofuran and 39-41 min for carbofuran. Conditions for long retention times were deliberately chosen because preliminary experiments with sample

extracts showed that most matrix interferences were weakly retained and eluted in the first part of the chromatogram.

The observed chromatographic behaviour upon coupling the precolumn to the analytical column is explained by the different nature of the two stationary phases. Due to its higher hydrophobicity and aromatic character, the polymeric adsorbent interacts more strongly with aromatic compounds than the C-18 phase (aliphatic); therefore the transfer of solutes from the former to the latter provokes widening, tailing and in some cases peak splitting, depending on the composition of the mobile phase. Acetonitrile compared to methanol is a stronger eluent in C-18 phases, but its relative strength is considerably higher in polymeric phases. Thus, the different retentiveness of PLRP-S and C-18 sorbents could be compensated to some extent using acetonitrile in the mobile phase.

Carbofuran and the phenolic metabolite present UV absorption maxima near 210 and 280 nm. Absorbance is higher at 210 nm but many organic compounds also present a high absorbance at this wavelength. Indeed, chromatograms obtained from the direct injection of a crude sample extract readily showed that the number and intensity of signals from the extracted matrix components were notably more important at 210 than at 280 nm. Therefore, the second absorption maximum of lower sensitivity but higher selectivity was chosen for detection.

**Sample Extraction**. Carbofuran and 7-phenolcarbofuran are quite soluble in polar and medium polarity solvents. Acetonitrile and methanol were assayed for the extraction of pesticides from the biological matrix because they are of moderate cost, readily available in high purity, and more selective than other solvents of lower polarity. Blank samples were extracted with identical volumes of the two solvents in an ultrasonic bath and the crude extracts were directly injected in the HPLC system. Comparison of chromatograms indicated that methanol was more selective as less matrix impurities were extracted with this solvent. However, chromatographic signals obtained with the methanolic extract still were too numerous and their absorbance was too high, in comparison with the peaks obtained from injection of a standard at low analyte concentrations.

**Preconcentration and Cleanup**. An 11-mL volume of methanolic extract was obtained from the previous step. It was further evaporated to 1 mL, diluted with water in a 1:1 ratio and slightly acidified with formic acid. This treatment provoked the formation of a precipitate, thus enabling elimination of a fraction of extracted impurities (probably compounds of high molecular weight and/or other organic molecules with acid-base properties which became neutral and less soluble upon addition of acid). The filtrate was diluted with more water to decrease the methanol content to less than 10% and was then loaded in the precolumn. Independent experiments demonstrated that the studied compounds were quantitatively retained in the precolumn under these conditions.

Upon percolation through the precolumn, the analytes and most matrix impurities remaining in the diluted extract were retained on the small sorbent bed and preconcentrated, but a fraction of coextracted compounds comprising inorganic salts and organic compounds of high polarity or ionized was eliminated in the effluent. Rinsing of the precolumn with 5% acetonitrile in water continued the elimination of the less hydrophobic compounds without eluting the compounds of interest. Further washing with a basic solution (pH 11) provoked ionisation and elution of an additional fraction of coextracted impurities. The last solution was rapidly replaced with the acetonitrile-water mixture (5:95, v/v) to avoid degradation of analytes and to protect the C-18 column from the basic media during the on-line elution of the precolumn.

Experimental conditions (volumes, composition, etc.) in the whole sample pretreatment process were stepwise adjusted and verified to ensure that analyte losses did not occurred. The final result is shown in the chromatograms of Figure 2. Two tissue portions (250 mg each) from the lot of blank samples were treated according to the developed procedure and analysed by HPLC-UV at 280 nm; one of them was fortified with 108 ng of each analyte and the other was a non-fortified tissue. As observed, the chromatographic profile is practically identical with the exception of the analyte peaks that are only present in the chromatogram of the spiked sample. Carbofuran (peak 2) and 7-phenolcarbofuran (peak 1) are well separated from each other and elute in a rather clean region without interference from the matrix peaks.

**Method Evaluation**. Four 250-mg portions of epithelial tissue (from the lot of blank samples) were spiked with different amounts of the pesticide and metabolite in the range 27-108 ng, which corresponds to concentrations of 0.11-0.43 μg/g (mass of analyte per gram of tissue). Results obtained from the analysis of samples according to the proposed method are shown in Table 1. As observed, the quantitation of both compounds was satisfactory with the exception of the sample

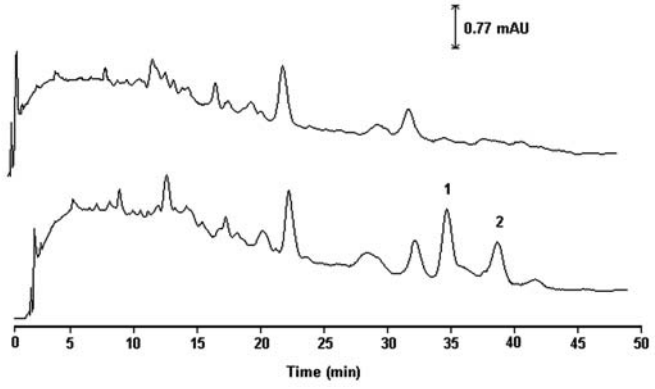


**Figure 2**. Chromatograms obtained from the analysis of 250-mg portions of epithelial tissue (blank samples). Upper trace: non-fortified sample; lower trace: sample fortified with 108 ng of each analyte. Solutes: (1) 7-phenolcarbofuran, and (2) carbofuran.

**Table 1**. Analysis of epithelial tissue samples (250 mg) spiked with carbofuran and 7-phenolcarbofuran at different concentrations.

| Compound | Spiked | | Recovered | | Recovery |
|---|---|---|---|---|---|
| | (ng) | (μg/g) | (ng) | (μg/g) | (%) |
| carbofuran | 27.1 | 0.108 | 35.1 | 0.140 | 129 |
| | 54.2 | 0.217 | 50.4 | 0.202 | 95 |
| | 81.3 | 0.325 | 80.6 | 0.322 | 99 |
| | 108 | 0.434 | 107 | 0.428 | 99 |
| 7-phenol carbofuran | 27.4 | 0.110 | 29.7 | 0.119 | 108 |
| | 54.8 | 0.219 | 51.4 | 0.206 | 94 |
| | 82.2 | 0.329 | 71.7 | 0.287 | 87 |
| | 110 | 0.438 | 101 | 0.404 | 92 |

spiked at the lowest concentration level where carbofuran was notably overestimated. This was not surprising because the peak size for analyte amounts near 20 ng approaches the level of the baseline noise. The calculated recoveries (excluding the lowest concentration) vary in the range 87-99%, which is generally considered a fairly good result in environmental analysis of trace pollutants with less complex matrices (i.e. water). Indeed, quality criteria to assess the performance of analytical methods for the determination of pesticides in biological matrices have not yet been established.

Due to the limited availability of samples, replicate analyses were only performed for one concentration level. Table 2 shows the statistic parameters calculated from results obtained in the analysis of seven 250-mg samples spiked with 54.2 ng (concentration 0.217 μg/g) of carbofuran and 54.8 ng (concentration 0.219 μg/g) of 7-phenolcarbofuran. For these analyte amounts, the method precision evaluated as relative standard deviation (%RSD) was 12.7% and 12.1%, and the method accuracy expressed as mean recovery (%R) was 91% and 97.8% for carbofuran and the metabolite, respectively.

Results in Table 2 were also used to estimate the limit of detection (LOD) and the limit of quantitation (LOQ) of this method. In the case of analytical methods for the determination of pollutants in water samples, EPA (US Environmental Protection Agency) recommends the use of the following equation to estimate LODs:

$$LOD = t_{(0.01,\ n-1)} \times SD \qquad \text{(E-1)}$$

Where, $t_{(0.01,\ n-1)}$ is the value of the Student "t" for a confidence level of 99% and n-1 freedom degrees; SD is the standard deviation (in concentration or mass units) obtained in the analysis of at least 7 blank samples spiked with the analyte at levels not exceeding 10 times the value of the expected LOD. Using the EPA recommendation (equation E-1) and the standard deviation reported in Table 2 with $n = 7$, the estimated method LOD for both compounds was 20 ng. This absolute LOD corresponds to a concentration of 0.08 μg/g in 250-mg tissue samples; however, if larger samples are analysed, a lower LOD in concentration units can be achieved.

On the other hand, a generally accepted criterion to estimate LOQs of analytical methods for environmental analysis does not exist. The standard deviation obtained in replicate analysis of a sample spiked at low concentration levels is sometimes used to calculate LOQs, according to the next equation:

$$LOQ = r \times SD \qquad \text{(E-2)}$$

Where, "*r*" is an arbitrary factor depending on the required precision (usually 3, 5 or 10). In this work, the method LOQ was estimated using equation E-2 with a factor $r = 5$, and the standard deviation reported in Table 2. The estimated LOQ for both compounds was approximately 32 ng (corresponding to a concentration of 0.13 μg/g for 250-mg samples).

**Application of the Method**. Three samples from women living in an agricultural region of Mexico were analysed according to the developed method. Some relevant details are given below.

Sample 1 (S-1): epithelial tissue with no anomalies detected by clinical analysis; sample weight 214.2 mg (wet basis).

Sample 2 (S-2): epithelial tissue with no anomalies, sample weight 622.9 mg (wet basis). The tissue was divided in three parts labelled S-2a (163.6 mg), S-2b (247.4 mg) and S-2c (211.9 mg). Each part was independently analyzed, but samples S-2b and S-2c were spiked with carbofuran (54.2 ng) and 7-phenolcarbofuran (54.8 ng) prior to analysis.

Sample 3 (S-3): cervical-uterine biopsy (cone) from a woman with *in situ* cancer, sample weight 928 mg (wet basis).

It is important to consider that the humid and warm conditions prevailing in the epithelial tissue of the uterus easily promote the progressive transformation of hydrolysable compounds. Therefore, eventual detection of carbofuran in a sample should necessarily be accompanied by detection of the phenolic metabolite. On the contrary, detection of 7-phenolcarbofuran in a sample does not necessarily implicate the simultaneous presence of carbofuran because total degradation

**Table 2**. Statistic parameters derived from the analysis of epithelial tissue samples (250 mg) spiked with carbofuran (54.2 ng, 0.217 μg/g) and 7-phenolcarbofuran (54.8 ng, 0.219 μg/g); n = 7.

| | Mean | | SD | | Precision | Accuracy |
|---|---|---|---|---|---|---|
| | (ng) | (μg/g) | (ng) | (μg/g) | (% RSD) | (% R) |
| carbofuran | 49.3 | 0.197 | 6.3 | 0.025 | 12.7 | 91.0 |
| 7-phenol carbofuran | 53.6 | 0.214 | 6.5 | 0.026 | 12.1 | 97.9 |

SD: standard deviation ; RSD: relative standard deviation ; R: recovery

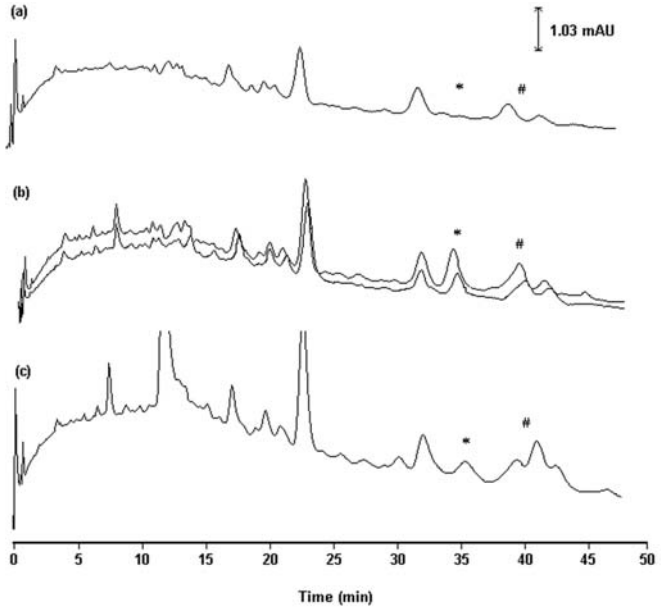


**Fig. 3**. Analysis of samples from countrywomen exposed to carbofuran. (a) epithelial tissue with no anomalies according to clinical analysis (sample S-1), (b) epithelial tissue with no anomalies according to clinical analysis; the lower trace corresponds to a non-fortified portion of this tissue (sample S-2a) and the upper trace is another portion of the same tissue fortified with carbofuran and 7-phenolcarbofuran, ~54 ng each (sample S-2b), and (c) cervical-uterine biopsy from a woman with *in-situ* cancer. The marks indicate the retention times of 7-phenolcarbofuran (∗) and carbofuran (#) determined from injected standards.

(hydrolysis, oxidation) of the parent compound could have occurred.

Figure 3 shows the chromatograms obtained from sample analysis. The two small marks on each graphic indicate the retention times of carbofuran (#) and 7-phenolcarbofuran (∗) as determined from injection of a standard just after the chromatographic run of the corresponding sample. It is observed that chromatographic profiles for the three samples are not identical and also differ from those previously obtained with blank samples (Fig. 2). This is normal considering the different origin of each tissue and the natural variability between individuals. However, all chromatograms in Fig. 3 present some peaks in the region where carbofuran elutes, while chromatograms from non-spiked blank samples were fairly clean in the same region. Because of these unexpected matrix interferences, quantitation of carbofuran in the studied samples was not carried out and only a tentative identification of the pesticide peak was assayed. On the other hand, the region where 7-phenolcarbofuran elutes is quite clean in all chromatograms; either no peaks, or only one peak is observed near the retention time mark.

Fig. 3a, corresponding to chromatogram of S-1, shows a flat baseline with no peaks at the retention time of 7-phenolcarbofuran. Although two peaks are observed in the neighbourhood of the carbofuran retention time, they are most probably due to matrix components because the hydrolysis product of the parent pesticide (*i.e.* 7-phenolcarbofuran) was not found. Therefore, no definitive evidence of contamination by the pesticide was detected in this tissue.

Fig. 3b shows chromatograms obtained from the analysis of S-2a (non-fortified sample, lower trace) and S-2b (fortified sample, upper trace). The chromatogram of S-2c (not shown) gave a similar profile. Two peaks eluting very close to the analyte retention time marks are observed in the chromatogram of the non-spiked sample; of course, the same peaks, but larger, appear in the fortified sample. It is important to notice that the peak near the (∗) mark has the same shape in both chromatograms and does not show any shoulder. Therefore, it is highly probable that only one compound, namely the metabolite, was responsible for this peak. On the other hand, the peak close to the (#) mark in the non-fortified sample chromatogram could be ascribed to carbofuran, but the peak purity and identity remain in doubt because of the matrix interferences eluting in this region.

Fig. 3c is the chromatogram of sample S-3. The peak eluting at the retention time of 7-phenolcarbofuran strongly suggests the presence of the metabolite in this tissue. Besides, there is a group of at least three partially overlapped peaks in the region where carbofuran elutes. Although the first peak of the group is closest to the retention time mark, it is not possible to affirm that it corresponds to the pesticide.

Quantitative results for the peak eluting at the same retention time as 7-phenolcarbofuran in samples S-2 and S-3 are presented in Table 3. The result for sample S-2 comes from addition of the metabolite amount determined in each portion of the divided tissue, after subtracting the spiked amount in samples S-2b and S-2c. As observed, the concentration of 7-phenolcarbofuran was about four times higher in sample S-2 than in sample S-3. This is an impacting result because S-2 came from a woman with no cervical-uterine anomalies according to the diagnostic issued from clinical analysis, while S-3 came from a woman with an *in situ* cancer. Whether carbofuran and/or the metabolite are cancer inductors cannot be deduced from these results. However, the highly probable presence of 7-phenolcarbofuran in two of the three examined samples is of concern and deserves a profound investigation on the health effects of carbofuran in this exposed population.

**Table 3**. Quantitative determination of 7-phenolcarbofuran in cervical-uterine epithelial tissue samples from exposed women.

| Sample | Weight (mg) | Metabolite Amount (ng) | Metabolite Concentration (μg/g) |
|---|---|---|---|
| S-2 | 623 | 179* | 0.29 |
| S-3 | 928 | 65 | 0.07 |

* Total amount obtained from the 3 tissue portions (S-2a + S-2b + S-2c) after subtracting the spiked amount in samples S-2b and S-2c.

The method presented in this work can be easily implemented in small local laboratories for a preliminary screening of samples. Positive results should be verified by alternative and more sophisticated analytical techniques, such as HPLC-MS (mass spectrometry) to confirm the identity of the peaks assigned to 7-phenolcarbofuran and carbofuran. The developed procedure including sample extraction, preconcentration and cleanup, as well as HPLC conditions, is also applicable with an MS detector.

## Conclusions

The high incidence of cervical-uterine cancer in country-women may be associated to a continuous contact with toxic pesticides such as carbofuran. The method proposed in this work can be used as a screening tool to detect the possible presence of carbofuran and/or 7-phenolcarbofuran in cervical-uterine biopsies from exposed women. Evaluation of the metabolite concentration is also feasible because no apparent interferences have been observed in the samples so far examined. However, depending on the sample, coextracted matrix impurities eluting near the retention time of carbofuran may be observed; therefore, unequivocal identification or correct integration of the pesticide peak are not always possible. Considering the complexity and variability of biological samples, confirmation of positive results by an alternative technique is recommended, as false positives are not completely excluded.

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