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*Full Length Research Paper*

# Rapid multi-residue method for the determination of pesticide residues in human serum

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**Exposure to pesticides can represent a potential risk to humans. Agricultural workers are at risk of chronic toxicity. Hence, the evaluation of pesticide residues in their blood gives an indication about the extent of exposure and help in assessing adverse health effects. The aim of our study was to develop analytical method for the simultaneous determination of some residues of pesticides using gas chromatography-mass spectrometry (GC-MS). This method involves a liquid-liquid extraction procedure. Pesticide residues were separated and detected using GC-MS, and acquisition was performed in the sellected ion monitoring (SIM) mode. For most of the pesticides, average recoveries ranged between 65 and 101% at three different fortification levels. The linearity of the method was satisfactory in the range of 5 to 50 ng/ml, with a correlation coefficient between 0.998 and 0.999, depending on the analyte. The estimated limit of detection and limit of quantification ranged from 2 to 5 ng/ml and from 5 to 10 ng/ml, respectively. The method precision and accuracy were found to be satisfactory at three concentration levels. The variation coefficients of intra-day and inter-day precision ranged from 0.4 to 14% and from 2.5 to 15%, respectively for most studied pesticides.**

**Key words:** Analytical methods, human serum, GC-MS, pesticide residues, validation.

## INTRODUCTION

The widespread use of pesticides has raised problems for both the environment and human health. Exposure to these hazardous compounds is of great concern among the general public. In general, humans are inevitably exposed to pesticides through environmental contamination or occupational use (Bolognesi, 2003). Therefore, exposure to pesticides represents a potential risk to humans, and some studies have shown associated harmful effects of these compounds on human health, such as an increase in endocrine, developmental, immune and neuropsychological disorders (Stephens et al., 1995; Parron et al., 1996; Mathur et al., 2002; Hernandez et al., 2003; Salvi et al., 2003), high probable risk of neurodegenerative diseases, particularly Parkinson's and Alzheimer diseases (Baldi et al., 2003; Kamel and Hoppin, 2004; Yih-Ru, 2005) as well as an increase in some types of cancer (Mathur et al., 2002; Bolognesi, 2003; Clary et al., 2003; Hernandez et al., 2003). In this way, according to the study of Russo et al. (2002), the analysis of human tissue samples (liver, healthy kidney, cancerous kidney and adipose tissues) for the determination of organophosphorus pesticides has indicated that concentrations of these compounds are more elevated in cancerous kidney samples than those detected in healthy kidney ones. Thus, biological monitoring is a useful tool for assessing exposure to pesticides and involves the measurement of a biomarker of exposure (usually the pesticide or its metabolite) in human blood, urine or tissues, thereby determining the internal dose of the toxicant (Aprea et al., 2002). As the general population is exposed to pesticides, it is important to investigate the

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concentration levels of pesticides and their metabolites in samples of human origins.

Owing to their chronic and occupational exposure to these compounds, agricultural workers are at a high risk of acute and chronic toxicity (Parron et al., 1996; Hernandez et al., 2005). Therefore, regular monitoring of health of these workers is necessary. The evaluation of pesticide residues in their blood gives an indication about the extent of exposure and helps in assessing adverse health effects (Aprea et al., 2002; Ramesh and Ravi, 2004).

Numerous analytical methods were previously developed for the determination of pesticide residues or their metabolites in human tissues (Mathur et al., 2002; Russo et al., 2002; Tsoukali et al., 2005) or biological fluids (serum, whole blood, urine) (Matos et al., 1998; Frias et al., 2001; Liu and Pleil, 2002; Ramesh and Ravi, 2004; Ariffin and Anderson, 2006). Chromatographic methods, particularly gas chromatography (GC) and liquid chromatography (LC), using various existing detection systems such as nitrogen-phosphorus detection (NPD) (Tarbah et al., 2001; Tsoukali et al., 2005), electron-capture detection (ECD) (Mathur et al., 2002; Heudorf et al., 2003) and mass spectrometry (MS) (Angerer et al., 1997; Hernandez et al., 2002; Schettgen et al., 2002; Ariffin and Anderson, 2006; Bouwman et al., 2006; Petropoulou et al., 2006; Inoue et al., 2007) were preferred for the analysis of these compounds. The majority of these previous studies were developed for forensic applications or diagnosis of acute pesticide intoxication. Furthermore, they were often described for the determination of pesticides from only one class such as carbamates, organochlorines, and especially organophosphorus and pyrethroids. However, agricultural workers are chronically exposed to pesticides, not only to one particular but most often simultaneously, to many mixtures of pesticides of many classes.

Hence, the aim of this work was to develop an analytical method for the determination of some pesticide residues widely used in agriculture (particularly in Tunisia), from several compound classes in serum, using gas chromatography-mass spectrometry (GC-MS). The application of the method was investigated in a population of agricultural workers of the Sahel region of Tunisia.

## MATERIALS AND METHODS

### Reagents

The chemicals and reagents used in this research were all of analytical grade. All pesticide pure standards were obtained from Cluzeau Info Labo (Sainte-Foy-la-Grande, France). Methanol was purchased from Carlo Erba Reagenti (Rodano, Italy). Ethyl acetate (Suprasolv, Merck), acetone, hexane, dichloromethane, (Pestinorm grade), acetic acid and sodium acetate were purchased from VWR (Fontenay sous-bois, France). A stock standard solution for each pesticide was prepared at 1 g/L in methanol.

The working solution was prepared by the dilution of a mixture of stock solution in acetone/dichloromethane/hexane (50/20/30, v/v/v) at the following concentration: 0.1, 1 and 10 mg/L. Mephenytoine used as internal standard (IS), was prepared at 20 mg/L in the same solvent mixture.

### Apparatus

The GC-MS system consisted of Shimadzu GC 2010 gas chromatograph equipped with a split/splitless injector, with an AOC 20i autosampler and coupled to Shimadzu QP 2010, quadropole mass spectrometer (Champs sur Marne, France), and a packed column inlet with glass sleeve using direct injection-port mode. The analytical column used was a 30 m x 0.25 m I.D. with 0.25 µm film thickness, PTE5 (Supelco, Isle d'Abeau, France) and coated with a 5% biphenyl-95% dimethylsiloxane stationary phase. The initial column temperature was set at 60°C for 2 min, and then increased at a rate of 15°C/min to 220°C. It was finally raised at a rate of 4°C/min until it got to 300°C and was maintained at this temperature for 1 min. The temperature of the injector, used in splitless mode, and of the transfer line were 250 and 280°C, respectively. Helium was used as the carrier gas at a constant flow-rate of 0.8 ml $min^{-1}$. The mass spectrometer was operated in electron ionisation mode (70 eV). The initial analysis was performed using full SCAN mode through which we acquired spectra and gleaned characteristic ions for the selected compounds. Quantification was performed on single ion monitoring (SIM) mode using the most abundant or characteristic mass fragment for quantification and two fragment ions for confirmation for each analyte (Table 1). These mass-to-charge ratios were carefully selected to avoid all those belonging to other pesticide residues of the same class. Pesticides analytes were subsequently identified by their relative retention time and by the ratios of their respective confirmation ions to their quantization ions.

### Sample collection

In the Sahel region of Tunisia, vegetables are among the most planted crops. Several kinds of pesticides are used to protect these crops, especially pyrethroids, organophosphorus and carbamates. According to the questionnaire filled by the agricultural workers of the region, before their blood samples were collected, it was impossible to determine the exact types and amount of pesticides used within a definite period of time, for each worker given the large number and mixture of pesticides used. Thus, in the present study, the pesticides selected were those that were most widely used in the Sahel region of Tunisia.

Blood samples, used for optimization and validation of the developed method were obtained from not-occupationally exposed healthy subjects, while analysed samples were collected from a population of agricultural workers chronically and occupationally exposed to pesticides in the region. All samples were centrifuged for 6 min at 3000 rpm, to separate the serum, and then stored at -80°C until they were analyzed.

### Extraction procedure

An aliquot of 2 ml of the serum sample was transferred into a 15 ml glass vial and spiked with 100 µL of IS solution. After addition of 1 ml of sodium acetate buffer (3M, pH 4.5) and 9 ml of the solvent mixture (acetone/dichloromethane/hexane) (50/20/30, v/v/v), the solution was mixed for 15 min and centrifuged at 3000 rpm for 5 min. The organic phase was evaporated to dryness under a gentle stream of nitrogen; the residue was re-dissolved in 140 µL of ethyl acetate and mixed thoroughly on vortex. The solution was then placed on ultrasound bath for 3 min and centrifuged at 3000 rpm for 5 min. Finally, 1 µL of this solution was injected into the GC-MS system.

**Table 1.** Retention times (RT), quantification and confirmation ions selected for GC-MS determination of pesticides in human serum.

| Pesticide | Retention time (min) | Quantitation ion (m/z) | Confirmation ion (m/z) |
|---|---|---|---|
| Dichlorvos | 9.08 | 109.0 | 184.9, 220.0 |
| Dimethoate | 13.37 | 87.0 | 125.0, 93.0 |
| Carbofuran | 13.41 | 164.0 | 149.0, 221.0 |
| Chlorothalonil | 14.20 | 265.9 | 263.9, 267.9 |
| Pirimicarb | 14.34 | 166.1 | 72.1, 238.0 |
| Chlorpyriphos-methyl | 14.82 | 285.8 | 287.9, 289.9 |
| Methalaxyl | 15.03 | 206.0 | 249.0, 279.0 |
| Dichlofluanid | 15.56 | 225.1 | 167.1, 223.9 |
| Chlorpyriphos-ethyl | 15.77 | 313.9 | 315.9, 285.8 |
| Folpet | 16.96 | 104.0 | 261.9, 259.9 |
| Dieldrin | 18.26 | 276.9 | 278.9, 345.0 |
| Propargite | 20.89 | 135.0 | 350.0, 173.0 |
| Iprodione | 21.55 | 314.0 | 316.0, 245.0 |
| Tetradifon | 22.91 | 355.9 | 353.9, 228.9 |
| Pirazophos | 24.41 | 221.0 | 232.0, 373.0 |

GC-MS, Gas chromatography-mass spectrometry.

**Validation of the method**

In order to construct calibration curves, 2 ml of human serum was spiked with the studied pesticides at final concentrations ranging from 5 to 50 ng/ml of serum and was analyzed with the described procedure. Linear calibration graphs were obtained by least squares regression of concentration versus area ratio of analyte to internal standard of the calibration samples.

The limit of detection (LOD) and limit of quantification (LOQ) were defined from spiked serum. LOD was defined as the lowest concentration of a pesticide giving a response of three-times the average of the baseline noise. LOQ was defined as the lowest concentration of pesticide that could be measured with relative standard deviation less than 20%.

The extraction recovery of the compounds were studied at three concentration levels (5 or 10, 30 and 50 ng/ml), by extracting the human serum spiked with different amounts of analytes, and then comparing the peak area ratio of each analyte to the internal standard in extracted samples with that in standard solution, prepared in blank sample extracts at the same concentration.

The intra and inter-day precision was assessed in triplicate at three concentration levels (5 or 10, 30 and 50 ng/ml). The intra-day precision was studied by the extraction and analysis of spiked samples on the same day, in each level concentration of fortified samples. For the inter-day precision, spiked samples were extracted and analysed each day for three days in triplicate, for each level concentration.

To study the specificity of the method, the existence of potential interferences in the chromatograms from the serum samples was monitored by running a blank sample in each calibration procedure.

## RESULTS AND DISCUSSION

A liquid-liquid extraction (LLE) method was applied for the extraction of studied pesticides. Preliminary experiments were carried out using the three organic solvents (acetone, dichloromethane and hexane), every one alone or in mixture with different proportions. Best recoveries for most pesticides studied were obtained using the mixture of the three solvents with the proportion of 50/20/30, respectively. A similar method previously reported by Lacassie et al. (1998) was developed for the simultaneous extraction of nine pesticides in fruits using this mixture of the three solvents. Thus, in spite of the complexity of the two matrices (fruits and human serum), this solvent mixture proved to be effective with a good extraction. This solvent mixture was suitable for both non-polar and slightly polar pesticides. Acetone and dichloromethane were sufficiently polar to extract a wide polarity range of pesticides, whereas, hexane assures the extraction of non-polar and moderately polar compounds and minimized the extraction of polar co-extractives (Lacassie et al., 1998; Van Der Hoff and Van Zoonen, 1999). Other studies involved also an LLE procedure and applied one solvent or a mixture of solvents for the extraction of pesticides from human biological samples (Frias et al., 2001; Liu and Pleil, 2002; Mathur et al., 2002; Russo et al., 2002; Schettgen et al., 2002; Heudorf et al., 2003; Ramesh and Ravi, 2004; Inoue et al., 2007). Most of them involved several steps and an important volume of hexane alone or in mixture with other organic solvents for the extraction of pesticides from blood samples (Angerer and Ritter, 1997; Frias et al., 2001; Liu and Pleil, 2002; Schettgen et al., 2002). In these studies, recoveries varied, from 40 to 128%, depending on the specific compound.

Each analyte was identified by comparing with its standard mass spectrum. The chromatogram of the studied standard pesticides is presented in Figure 1. Quantification was performed by monitoring individual ions, for each selected pesticide using SIM mode. Retention times (RT), quantification and confirmation ions selected for each studied pesticide are shown in Table 1.

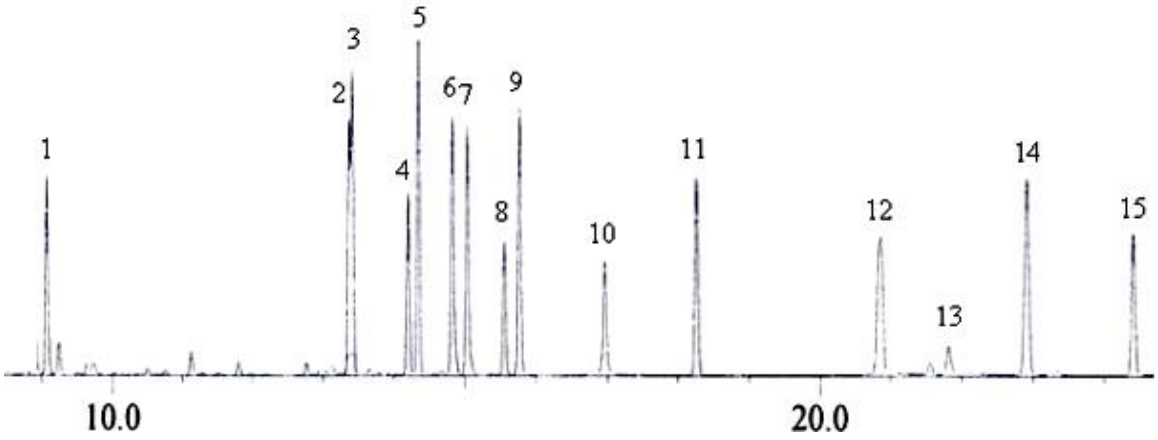


**Figure 1.** GC-MS chromatogram (full-scan mode) of pesticides standard mixture (10 µg/ml). Peaks: 1, dichlorvos; 2, dimethoate; 3, carbofuran; 4, chlorothalonil; 5, pirimicarb; 6, chlorpyriphos-methyl; 7, methalaxyl; 8, dichlofluanid; 9, chlorpyriphos-ethyl; 10, folpet; 11, dieldrin; 12, propargite; 13, iprodione; 14, tetradifon; 15, pirazophos. GC-MS, Gas chromatography-mass spectrometry.

**Table 2.** Average extraction recoveries (%,±RSD) (n=3) of pesticides analysed in human serum by GC-MS, at three concentration levels.

| Pesticide | 5/10 (µg/L) | 30 (µg/L) | 50 (µg/L) |
|---|---|---|---|
| Dichlorvos | 61.40 ± 1.90 | 56.59 ± 2.43 | 78.77 ± 4.44 |
| Dimethoate | 69.40 ± 5.76 | 61.26 ± 0.84 | 92.52 ± 9.00 |
| Carbofuran | 85.04 ± 7.55 | 65.65 ± 3.90 | 88.10 ± 15.24 |
| Chlorothalonil | 74.32 ± 11.11 | 49.80 ± 0.62 | 49.47 ± 5.70 |
| Pyrimicarb | 94.65 ± 17.62 | 71.12 ± 0.78 | 82.54 ± 11.92 |
| Chlorpyriphos-methyl | 65.51 ± 6.73 | 59.97 ± 0.10 | 86.03 ± 12.00 |
| Methalaxyl | 81.53 ± 12.59 | 68.41 ± 1.29 | 84.10 ± 11.02 |
| Dichlofluanid | 92.69 ± 18.61 | 63.42 ± 0.67 | 60.26 ± 1.64 |
| Chlorpyriphos-ethyl | 70.38 ± 3.63 | 58.75 ± 3.32 | 79.39 ± 8.81 |
| Folpet | 68.82 ± 10.78 | 67.85 ± 0.54 | 96.00 ± 7.71 |
| Dieldrin | 94.29 ± 4.28 | 79.58 ± 3.74 | 82.64 ± 7.55 |
| Propargite | 69.85 ± 7.03 | 90.52 ± 2.31 | 85.01 ± 1.81 |
| Iprodione | 95.17 ± 9.64 | 82.49 ± 1.66 | 90.19 ± 0.79 |
| Tetradifon | 96.39 ± 3.99 | 92.51 ± 2.49 | 97.27 ± 0.17 |
| Pirazophos | 81.41 ± 11.62 | 77.97 ± 1.85 | 77.39 ± 4.42 |

GC-MS, Gas chromatography-mass spectrometry.

The results of our validation procedure are summarized in Tables 2, 3 and 4. Recovery of extraction procedure was studied at the three concentration levels. The lower concentration level studied corresponds to the LOQ of the method and it was 5 or 10 ng/ml depending on the target compound. The average recoveries were determined for each target compound at each concentration level (Table 2). Extraction recovery varied, depending on the pesticides studied, but was satisfactory with RSD values (that is, below 15% for most workers). The average recovery was higher than 60% at the three concentration levels studied except for dichlorvos and chlorothalonil for which recoveries at 30 ng/ml were 56.6 and 49.8%, respectively. According to Inoue et al. (2007) the supernatant which result, after extraction must not be evaporated to dryness under a stream of nitrogen gas because some pesticides, such as dichlorvos, are thermally unstable. Although we performed this evaporation step, in the present study, the average recoveries of dichlorvos were acceptable at 10 ng/ml (61.40%) and at 50 ng/ml (78.77%), but recoveries could be better if the evaporation to dryness was not performed for this compound. Therefore, the development of multi-residue methods is difficult, due to the fact that compounds of

**Table 3.** Correlation coefficients (r), limits of detection (LOD) and limits of quantification (LOQ) of studied pesticides in human serum.

| Pesticide | r | LOD (µg/l) | LOQ (µg/l) |
|---|---|---|---|
| Dichlorvos | 0.9998 | 5 | 10 |
| Dimethoate | 0.9999 | 2 | 5 |
| Carbofuran | 0.9995 | 2 | 5 |
| Chlorothalonil | 0.9998 | 5 | 10 |
| Pirimicarb | 0.9987 | 2 | 5 |
| Chlorpyriphos-methyl | 0.9980 | 2 | 5 |
| Methalaxyl | 0.9990 | 2 | 5 |
| Dichlofluanid | 0.9998 | 5 | 10 |
| Chlorpyriphos-ethyl | 0.9991 | 2 | 5 |
| Folpet | 0.9999 | 5 | 10 |
| Dieldrin | 0.9994 | 2 | 5 |
| Propargite | 0.9988 | 2 | 5 |
| Iprodione | 0.9990 | 5 | 10 |
| Tetradifon | 0.9993 | 5 | 10 |
| Pirazophos | 0.9985 | 5 | 10 |

**Table 4.** Precision (%) (n=3) of studied pesticides at three concentration levels in human serum.

| Pesticide | Intra-day precision (%) | | | Inter-day precision (%) | | |
|---|---|---|---|---|---|---|
| | 5/10 µg/L | 30 µg/L | 50 µg/L | 5/10 µg/L | 30 µg/L | 50 µg/L |
| Dichlorvos | 1.34 | 1.75 | 8.19 | 6.86 | 4.97 | 5.93 |
| Dimethoate | 10.80 | 3.29 | 1.93 | 16.26 | 4.64 | 5.87 |
| Carbofuran | 1.55 | 3.38 | 3.51 | 17.09 | 9.01 | 7.77 |
| Chlorothalonil | 11.10 | 1.36 | 14.55 | 14.99 | 10.35 | 16.76 |
| Pirimicarb | 0.78 | 6.16 | 2.77 | 16.35 | 15.78 | 9.64 |
| Chlorpyriphos-methyl | 3.03 | 2.94 | 9.06 | 15.31 | 11.33 | 6.51 |
| Methalaxyl | 4.24 | 4.69 | 2.41 | 14.71 | 13.15 | 9.14 |
| Dichlofluanid | 3.94 | 2.85 | 7.16 | 5.62 | 8.30 | 4.08 |
| Chlorpyriphos-ethyl | 5.25 | 0.57 | 7.16 | 8.63 | 7.46 | 5.37 |
| Folpet | 4.39 | 4.43 | 2.36 | 17.81 | 7.07 | 9.15 |
| Dieldrin | 5.43 | 7.46 | 2.76 | 8.63 | 3.71 | 7.26 |
| Propargite | 5.12 | 19.52 | 3.19 | 15.30 | 3.66 | 4.72 |
| Iprodione | 0.37 | 8.14 | 3.66 | 17.57 | 9.48 | 9.81 |
| Tetradifon | 8.53 | 5.08 | 9.45 | 8.56 | 3.46 | 6.85 |
| Pirazophos | 10.18 | 11.48 | 3.59 | 14.24 | 5.94 | 14.10 |

different physico-chemical characteristics, polarities, solubility and volatilities have to be simultaneously extracted and analysed.

The linearity of the calibration curves was studied for each pesticide, considering the area of peaks relative to IS. The correlation coefficient for each selected pesticide is listed in Table 3. Good linearity was found in the studied range with correlation coefficients between 0.9980 and 0.9999.

The sensitivity of the method was evaluated by determining the LOD and LOQ (Table 3). The criteria used for fixing the LOD are highly variable in literature. According to the review of analytical methods for biological monitoring of pesticide exposure described by Aprea et al. (2002), LOD of analyses vary widely and LOD higher than 1 ng/ml may be adequate for monitoring occupationally exposed workers. In the present study, LOD of all pesticides was between 2 and 5 ng/ml depending on the target compound. However, for monitoring the general population, LOD of the method must be lower than 1 ng/ml (Aprea et al., 2002).

The specificity of the method was demonstrated by the

absence of any chromatographic components at the same retention time as target pesticides in the blank serum samples. This finding suggests that no interferences from matrix occurred.

The results of precision at the three different concentration levels are summarised in Table 4. The variation coefficients of intra-day precision ($CV_r$) were found to be lower than 15% for all studied pesticides except for propargite for which the calculated $CV_r$ at concentration level of 30 ng/ml was 19.52%. Whereas, regarding the inter-day precision, the variation coefficients ($CV_R$) were higher than 15% for some studied compounds such as for folpet (17.81) and iprodione (17.57) at the concentration level of 10 ng/ml and chlorothalonil (16.76) at the concentration level of 50 ng/ml. For other compounds, the $CV_R$ were lower than 10% at the three different concentration levels. These values indicate that the present method is repeatable and reproducible.

The application of the present method was investigated in a population of agricultural workers chronically and occupationally exposed to pesticides in the Sahel region of Tunisia. Blood samples were collected randomly from different age groups. The non-availability of the residues for analyzed samples may be due to the metabolism and the excretion of the compounds. Hence, the analysis of urine samples of these workers is required in order to screen metabolites of target compounds. Further research on metabolism of pesticides in occupationally exposed humans and the excretion of metabolites from the body is warranted.

## Conclusion

The present analytical method was developed for the simultaneous determination of pesticides of various compound classes in human serum samples. It involved a simple liquid-liquid extraction procedure and gas chromatography-mass spectrometry detection. The method specifications indicate that this method is reliable with satisfactory results. This method was linear in the studied range with correlation coefficients between 0.998 and 0.999. The LOD of all pesticides was between 2 and 5 ng/ml, depending on the target compound. The variation coefficients of intra-day and inter-day precision, evaluated at three concentration levels, were lower than 15% for most studied pesticides. Therefore, this method is suitable for monitoring the residue levels in agricultural workers, chronically and occupationally exposed to some common pesticides, in order to evaluate exposure extent and health risk of these workers.

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