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# Application of Liquid Chromatography with Diode-Array Detector for Determination of Acetamiprid and 6-chloronicotinic Acid Residues in Sweet Cherry Samples

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**SUMMARY**

A rapid and simple method for simultaneous determination of acetamiprid and its metabolite 6-chloronicotinic acid in sweet cherry samples has been developed. This residue analysis method is based on the reversed phase separation on $C_{18}$ column with gradient elution. Analytes' determination and quantification were performed by high performance liquid chromatography (HPLC) with diode-array detector and chromatograms were extracted at 230 nm. Extraction efficiency experiments demonstrated the ability of this method to extract neonicotinoids from sweet cherry samples. These insecticides were extracted with a mixture of acetonitril/0.1N ammonium-chloride (8/2, v/v). The average recoveries of acetamiprid and 6-chlornicotinic acid from sweet cherry samples were in the range of 95-101% and 73-83%, respectively, with the associated relative standard deviations (RSDs) <5%. Expanded measurement uncertainties for the analyzed compounds were 2.7 and 3.01%. The limit of quantification (LOQ) was 10 µg/kg and 30 µg/kg for acetamiprid and 6-chloronicotinic acid, respectively. Thus, the developed HPLC/DAD method can be considered a useful tool for sensitive and rapid determination of acetamiprid and 6-chloronicotinic acid. Hence, the method may find further application in the analysis of real sweet cherry samples contaminated with these insecticides at a ppb level.

**Keywords:** Acetamiprid; 6-chloronicotinic acid; HPLC/DAD; Sweet cherry; Pesticide residues

## INTRODUCTION

In modern agriculture, pesticides have been broadly employed in order to protect agricultural products against harmful insects and weeds, to improve their quality and increase their yields (Kim et al., 2008; Lee et al., 2008). Pesticide application which is not in accordance with GAP (Good Agricultural Practice) and ignoring the pre-harvest interval, leads to consequences for human health, beneficial insects and animals. For this reason there is a constant need to develop new and more sensitive analytical methods for quantitative determination and monitoring of pesticides in food and the environment. This is particularly true of fruits and vegetables which are mainly consumed fresh, such as sweet cherries.

In order to reduce the use of organophosphates for protection of cherries, alternative compounds have been recommended, such as those from the class of neonicotinoids. Over the past 15 years neonicotinoids have gained increasing interest in the agricultural sector across Europe (Council Directive 91/414/EEC). These insecticides are the fastest growing class of insecticides introduced to the market since the launch of pyrethroids (Muccio et al., 2006).

Neonicotinoids are used for foliar and soil application but imidacloprid also has a very high usage as a seed treatment (Roberts and Hutson, 1999). Neonicotinoid insecticides are strong selective agonists of insect nicotinic acetylcholine receptors (nAChRs), they exhibit specific activity against the insect nervous system. This unique mode of action makes these pesticides highly applicable for controlling the biological effect of insects in cases when they have developed resistance to organophosphate, carbamate and pyrethroid insecticides (Jeschke et al., 2001).

Besides their positive effects, neonicotinoid pesticides also pose various health risks to consumers. Due to a growing use of insecticides from the family of neonicotinoids, their increased presence in the environment is evident. For this reason, the concentration of neonicotinoid residues, including their metabolites, in agricultural products should be monitored. This requires appropriate methods of extraction and determination, such as the already existing methods for imidacloprid in potato and onion (Mandić et al., 2002, 2005; Lazić et al., 2002, 2003, 2004), or thiamethoxam in pepper (Lazić et al., 2002a).

Acetamiprid with the IUPAC name (E)-$N^1$-[(6-Chloro-3-pyridyl)methyl]-$N^2$-cyano-$N^1$-methylacetamidine, CAS number 135410-20-7 and molecular weight of 222.7 g/mol is a systemic insecticide. It is used to control Hemiptera, especially aphids, Thysanoptera and Lepidoptera on a wide range of crops, especially vegetables, fruits and tea (Roberts and Hutson, 1999). Its chemical structure is shown in figure 1.

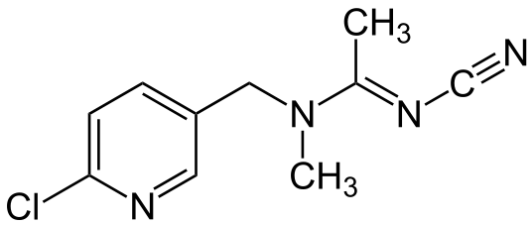


**Figure 1.** Chemical structure of acetamiprid

Following the legislation for food safety in vegetables and fruits, only parent insecticides (e.g. organophosphorus or neonicotinoid) are monitored and there is no control over the presence of metabolites or transformation products forming after application (Žabar et al., 2011). Based on a few published papers, a common transformation product of acetamiprid is 6-chloronicotinic acid (6CNA) (Marin et al., 2004). Its chemical structure is shown in figure 2.

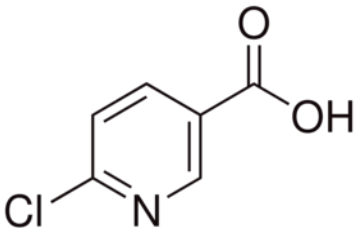


**Figure 2.** Chemical structure of 6-chloronicotinic acid (6CNA)

Determination of low concentrations of these pesticides in different matrices requires an effective fruit extraction procedure, followed by final chromatographic determination, in order to separate as much analyte as possible from the matrix interference substances. Gas chromatography (GC) combined with a selective and sensitive electron capture detector (ECD) and nitrogen phosphorus detector (NPD) has become a routine technique for the analysis of pesticides in fruits. Until now,

several analytical methods have been published for determining acetamiprid in crops and soils by GC/ECD (Tokieda at al., 1998, 1999), by high-performance liquid chromatography (HPLC) (Tokieda et al., 1998a) and by enzyme-linked immunosorbent assay (ELISA) (Wanatabe et al., 2000). However, the high sensitivity of these detectors contrasts with their lack of identification power. Relative retention-time-based identification and determinations of pollutants are no longer sufficient. An additional confirmatory technique is necessary, and detection by mass spectrometry (MS) is frequently used because of its identification capability. Acetamiprid and its metabolite 6CNA can also be determined using first-order derivative spectrometry (Gaál et al., 2005; Guzsvány et al., 2012; Guzsvány et al., 2012a).

There are also some alternative analytical approaches to the conventional methods of pesticides determination (Guzsvány, 2006; Papp et al., 2009; Guzsvány et al., 2012).

A number of papers have so far focused on determination of acetamiprid residues in cotton, vegetables, fruits, milk, honey and fine airborne particulate matter (Obananet al., 2002; Jansson et al., 2004; Ferrer et al., 2005; Fidente et al., 2005; Guzsvány et al., 2006a; Lesueur et al., 2008; Seccia et al., 2008; Zhang et al., 2008; Coscollà et al., 2009; Lee et al., 2009). In order to track the pesticide's metabolites in the environment and in different matrices, sensitive techniques for their monitoring should be developed. In literature, the most widely applied method is the mass spectrometry technique, coupled with GC or LC, because of its intrinsic characteristics, such as selectivity, sensitivity and identification-confirmation capability (Martínez Vidal et al., 2009; Hernández et al., 2008b; del Mar Gómez-Ramos et al., 2011). 6CNA has been determined in samples such as water, soil, air, honeybee, apple, potato, grape, bananas and maize (Gil García et al., 2007; Kamel, 2010; Žabar et al.,2012).

The principal objective of this study was to develop a simple and sensitive method for extraction and determination of acetamiprid and 6-chlornicotinic acid in sweet cherry samples using HPLC/DAD.

## MATERIAL AND METHODS

### Standards and reagents

Analytical standards of acetamiprid (98.1%) and 6-chloronicotinic acid (99.5%) produced by Dr Ehrenstorfer (Augsburg, Germany) were used. Acetonitrile (ACN) and dichloromethane of a suitable grade for pesticide residue analysis were from J.T. Baker, Germany. Reagent-grade ammonium chloride (in crystal form) was ordered from Alkaloid, Skopje. Sodium hydroxide (NaOH) was obtained from Hemos, Beograd. Ultra-pure water was obtained from the water purification system TKA, Germany. Sweet cherry samples were purchased from orchards untreated with pesticides, and stored at -10°C.

### Instrumental and Chromatographic Conditions

An HPLC method with UV detection was selected for the method of analysis. Chromatographic separation was carried out on an Agilent HPLC 1100 system (Agilent, United States) using a reversed phase procedure that utilized an Agilent Zorbax $C_{18}$ column (50 mm × 4.6 mm internal diameter, 1.8 μm particle size) and UV detection at 230 nm. This wavelength was selected because it is a UV maximum and provides the sensitivity needed for quantization of low concentration in the dissolution samples. The column temperature was maintained at 30°C. The mobile phases were acetonitrile (A) and aqueous 2% acetic acid (B). The gradient program was as follows: from 40-50% A in 2 min, from 50-40% A in 1 min. The total running time was 3 min and 1 min for re-equilibration after each analysis. The flow rate was constant, 1 ml/min during the whole process and 10 μl of sample was injected in every case.

### Standard solutions

A stock solution of each pesticide (acetamipride and 6-chloronicotinic acid) was prepared dissolving pure standard in a mixture of acetonitrile/water (ACN/$H_2O$). Working standard solutions containing a mixture of the analytes at concentration of 0.05–1 μg/ml were prepared from the above by appropriate solvent dilutions. Working standard solutions were used to calibrate the HPLC/DAD system and spike samples of sweet cherry in recovery experiments. All the solutions were protected from light and kept in a refrigerator until being used. All calibration standard solutions were prepared within 24 h of sample extract analysis. Figure 3 shows the chromatogram of the mixture of acetamipride and 6CNA.

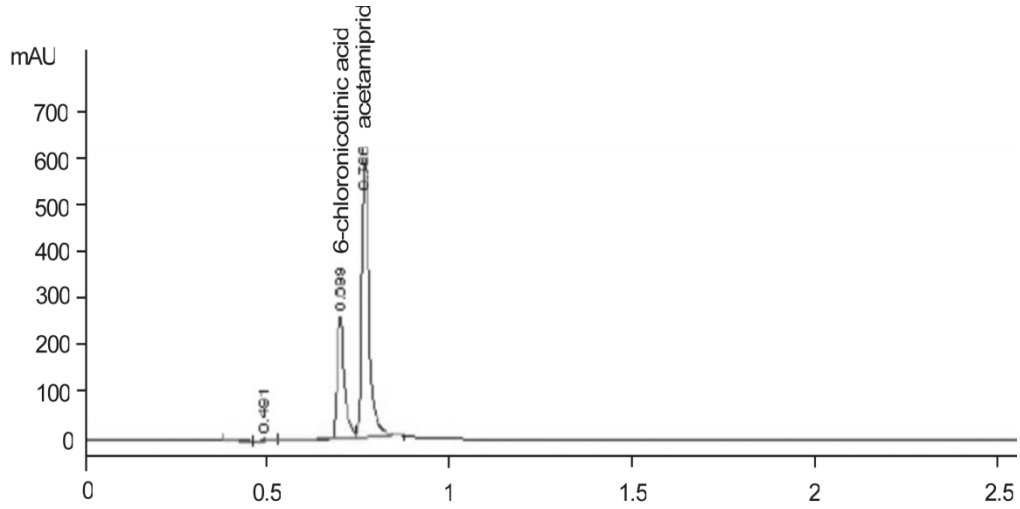


**Figure 3.** Chromatogram of the mixture of acetamipride and 6CNA

### Extraction procedure

Fresh samples of sweet cherry were homogenized with 20 ml volume of mixture of ACN and 0.1N ammonium-chloride, and vigorously mixed for 30 min using a shaker (Promax 2020, Heildolph). The fruit puree was clarified by centrifugation at 8000 rpm for 2 min. The supernatant was filtered through celite layer (1 cm) under vacuum and evaporated to ca. 5 ml using a rotary evaporator (Laborota 4000, Heildolph) with a water bath at maximum 40°C. The total volume of 5 ml after evaporation was dissolved in deionized water (pH 7 with NaOH). As previously mentioned, the whole mixture was added to 100 ml dichloromethane in the separatory funnel and vigorously mixed for 5 min. This step was repeated three times. After separating the layers, the whole dichloromethane layer was collected and evaporated to dryness. The dry residue was dissolved in 1 ml of ACN/$H_2O$ and analyzed by HPLC/DAD. Sweet cherry samples were fortified at three levels (0.2, 0.5 and 1.0 mg/kg) with a mixture of acetamiprid and 6CNA.

## RESULTS AND DISCUSSION

### Method validation

Analytical parameters related to linearity, repeatability, accuracy and limit of quantification (LOQ) were investigated to evaluate the viability of the proposed method. The linearity of the method was evaluated by constructing five-point calibration curves (each level in triplicate) with a wide concentration range (0.05-1 μg/ml). As shown in Table 1, good linearity was observed for both compounds at concentrations within the tested interval, with correlation coefficients ($R^2$) of 0.995 for acetamiprid and 0.947 for 6CNA.

The method's repeatability was tested by determining the RSD of chromatographic signals obtained from a mixture of standard solution of acetamiprid and 6CNA (concentration 0.1 μg/ml) analyzed five times. The repeatability of the retention times and peak areas of both compounds were tested. Retention time (Rt) of acetamiprid and 6CNA was 0.789 min and 0.699 min, respectively. The obtained values of relative standard deviation (RSD) of the retention times are shown in Table 1, while RSD of the peak area ranged from 0.84% for acetamiprid to 2.16% for 6-chloronicotinic acid. The RSD values achieved in these linearity and repeatability tests, indicating good precision of this method, are consistent with the regulations for analysis of pesticide trace levels (SANCO/12495/2011).

Limits of quantification (LOQ) for the analyzed acetamiprid and 6CNA were estimated from the fortified samples. Signal-to-noise (S/N) ratios reported by the instrument software were used to calculate the analyte concentration that yielded a signal-to-noise ratio of 10 times. The range of LOQs, summarized in Table 2, was from 10 μg/kg for acetamiprid to 30 μg/kg for 6-chloronicotinic acid. Precision at limit of quantification was checked by analyzing six test solutions prepared at LOQ level and calculating the percentage of RSD of area.

**Table 1.** Analytical parameters for HPLC/DAD determination of acetamiprid and 6CNA

| Parameter | Retention time (min), tr ± Δtr | Concentration interval (μg/ml) | Calibration curve | Linearity ($R^2$) |
|---|---|---|---|---|
| Acetamiprid | 0.789 ± 0.001 | 0.05-1 | y = 2.79+7.22x | 0.995 |
| 6CNA | 0.699± 0.002 | 0.05-1 | y = -1.53+5.56x | 0.947 |

The European Union has specified the maximum residue level (MRL) of 0.5 mg/kg for acetamiprid in sweet cherry (Regulation No. 978/2011 of the European Parliament and Council, 2011). The permissible level for acetamiprid in sweet cherry set by Serbian legislation is 0.2 mg/kg (Official Gazette, No. 25/2010). On the other hand, there is no control over the presence of pesticide transformation products forming after application (Žabar et al., 2011). The LOQs established for identification and quantification of target analytes were below the MRLs established by the EU legislation and Serbian Regulations.

Recovery was obtained for acetamiprid and 6CNA spiked at the appropriate concentration levels (0.2, 0.5 and 1.0 mg/kg) in sweet cherry samples. The obtained values of the recovery study for each compound are shown in Table 2.

**Table 2.** Recovery values and LOQ for determination of acetamiprid and 6CNA in sweet cherry samples

| Parameter | LOQ | Recovery ± RSD |
|---|---|---|
| Analyte | μg/kg | % |
| Acetamiprid | 10 | 97.5 ± 3.1 |
| 6CNA | 30 | 80.0 ± 4.1 |

This shows that mean recoveries ranged from 95-101% for acetamiprid and from 73-83% for 6CNA, with the associated relative standard deviations (RSDs) of 3.1% and 4.1% for acetamiprid and 6CNA, respectively. The RSDs were acceptable for analytical performance.

## Measurement uncertainty

In the method validation procedure, the estimation of uncertainty is one of the main focuses of interest due to its importance in showing the data quality. The ISO standard 17025 requires presentation of uncertainty data for analytical results. Since a typical chemical measurement consists of a number of measurement steps, it requires a careful design of measurement procedure to keep the traceability chain to the SI unit. To make a measurement result traceable to the SI unit, it is also necessary to evaluate the uncertainty of each step in the measurement procedure and combine them to meet the principles of the internationally agreed guide (Serpil, 2006; ISO/IEC17025).

According to the requirement of ISO17025, testing laboratories shall have and apply procedures for estimating the uncertainties of measurement. When estimating the uncertainty of measurement, all uncertainty components which are of importance in the given situation shall be taken into account using appropriate methods of analysis (ISO/IEC17025).

Measurement uncertainty is a quantitative indicator of confidence in the analytical data and describes the range around a reported or experimental result within which the true value can be expected to lie within a defined probability (confidence level). Uncertainty ranges must take into consideration all sources of error (SANCO/12495/2011).

The main sources of uncertainty were identified as uncertainty associated with the method of calibration, trueness of the method (recovery) and overall repeatability of the procedure. Uncertainty sources were further divided to uncertainty components: uncertainty of volumetric operations, uncertainty of chromatographic operations, purity of reagents and repeatability of operations. Measurement uncertainty evaluation was based on method validation data, assuming that they comprise the total analytical procedure. Trueness of the method was determined by a recovery study. The precision of the procedure represents a substantial source of measurement uncertainty and therefore requires detailed consideration in order not to lead to overestimation or underestimation of the combined uncertainty. With a combination of recovery, within-laboratory reproducibility and repeatability, all the relevant uncertainty sources were covered. The relative uncertainty of the recovery of the analyte was independent of concentration level. The uncertainty of standard addition

to a sample was calculated from the uncertainty of added volume and uncertainty arising from the concentration of acetamipride and 6-chlornicotinic acid solution. The limits of accuracy of the glassware were declared by the manufacturer. There were no data on distribution, so a triangular distribution was assumed. Therefore, to obtain the standard deviation, values were divided by $\sqrt{6}$ . Purity of the reagents acetamiprid and 6-chlornicotinic acid were evaluated according to the manufacturer's certificate. Since there were no data on distribution, a rectangular distribution was assumed, and therefore the stated uncertainty was divided by $\sqrt{3}$ for conversion to one standard deviation.

Standard uncertainty associated to bias (B) was calculated using the equation:

$$u(B) = \sqrt{B^2 + \frac{S_B^2}{\sqrt{n}} + u(C_R)}$$

where $B$ is deviation from true value, $S_B$ is standard deviation of the bias, $n$ is the number of measurements, $u(C_R)$ is uncertainty of the recovery. Uncertainty originating from method bias, within-laboratory reproducibility and repeatability was calculated to be 1.7, 0.2 and 0.6%, respectively. For the sweet cherry matrices studied, the combined relative uncertainty $u(CIMS)$ for acetamiprid and 6-chlornicotinic acid was 1.35% and 1.50%, respectively. The expanded uncertainty ($Uc$) was calculated as $Uc = k^*u_c$, where k is the coverage factor with a level of confidence of approximately 95% considering a coverage factor of 2 (Ellison et al., 2000). Expanded uncertainty $(Uc)$ for acetamiprid and 6-chlornicotinic acid were calculated to be 2.7% and 3.01%, respectively.

In this paper a possibility of simultaneous determination of acetamiprid and 6-chloronicotinic acid using HPLC/DAD in sweet cherry samples was developed. Considering the obtained values of analytical parameters, the proposed method proved to be an efficient and sensitive method for determination of these compounds in carry samples. Bearing in mind that the maximum residue levels of acetamiprid in cherries are 0.2 mg/kg and 0.5 mg/kg, the method is sensitive enough for determination of pesticides and their metabolites at concentrations well below permissible levels.

We proved that the method was specific for determination of acetamiprid and 6-chlornicotinic acid in the relevant matrices. After validation and measurement uncertainty evaluation steps, the results obtained showed that the method can be efficiently applied for monitoring of these compounds in sweet cherry samples.

## ACKNOWLEDGEMENT

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# Primena tečne hromatografije sa DAD detektorom za određivanje ostataka acetamiprida i 6-hlornikotinske kiseline u uzorcima trešanja

**REZIME**

U radu je predstavljena jednostavna metoda za određivanje acetamiprida i njegovog metabolita, 6-hlornikotinske kiseline, u uzorcima trešanja. Metoda je bazirana na primeni reverzno-faznog razdvajanja na C18 koloni primenom gradijentnog eluiranja. Određivanje i kvantifikacija analita je vršena tečnom hromatografijom (HPLC) sa DAD detektorom, pri čemu je korišćena talasna dužina od 230 nm. Tačnost metode je ocenjena procenom merne nesigurnosti. Ekstrakcija acetamiprida i 6-hlornikotinske kiseline iz uzoraka trešanja je vršena smešom acetonitril/amonijum-hlorid (0,1N) u odnosu 80:20 (v/v). Sva merenja su vršena u tri ponavljanja, pri čemu su dobijeni prinosi određivanja acetamiprida i 6-hlornikotinske kiseline u rasponima 95-101% i 73-83%, respektivno. Relativne standardne devijacije (RSD) merenja su u svim slučajevima bile ispod 5%. Limiti kvantifikacije za acetamiprid i 6-HNK iznosili su 10 i 30 µg/kg, respektivno. Kombinovana merna nesigurnost rezultata analize acetamiprida i njegovog metabolita procenjena je na 1,35, odnosno 1,50%, a proširena na 2,7 i 3,01%, upotrebom faktora pokrivanja (k=2) koji odgovara nivou poverenja od 95%, za normalnu raspodelu. Nakon validacije i procene merne neizvesnosti dobijeni rezultati pokazuju da se razvijena HPLC/DAD metoda može primeniti za određivanje sadržaja acetamiprida i 6-hlornikotinske kiseline u uzorcima trešanja i relevantnim matriksima kontaminiranim ovim jedinjenjima.

**Ključne reči:** Acetamiprid; 6-hlornikotinska kiselina; HPLC/DAD; trešnje; ostaci pesticida