ELSEVIER

Journal of Chromatography A, 835 (1999) 113–120

JOURNAL OF CHROMATOGRAPHY A

High-performance liquid chromatographic determination of benzoylurea insecticides residues in grapes and wine using liquid and solid-phase extraction

George E. Miliadis[a], Nicholas G. Tsiropoulos[b,*], Pipina G. Aplada-Sarlis[a]

[a]*Benaki Phytopathological Institute, 7 Ekalis Street, Kifissia 14561, Greece*

[b]*Department of Theoretical and Applied Sciences, University of Thessaly, Pedion Areos, Volos 38334, Greece*

Received 28 September 1998; received in revised form 14 December 1998; accepted 30 December 1998

Abstract

A method for the determination of the benzoylurea insecticides diflubenzuron, triflumuron, teflubenzuron, lufenuron and flufenoxuron in grapes and wine by HPLC has been developed and validated. Grape samples (50 g) were homogenized and extracted with ethyl acetate–sodium sulfate and further cleaned-up by solid-phase extraction on silica sorbent. Wine samples (10 ml) diluted with water (1:3) were solid-phase extracted on an octadecyl sorbent using methanol as the eluent. The pesticides were separated on a reversed-phase octadecyl narrow-bore column by gradient elution and the residues were determined with a UV diode array detector. The calibration plots were linear over the range 0.05–5 μg/ml. Recoveries of benzoylurea pesticides from spiked grapes (0.02–2.0 mg/kg) and wine (0.01–0.2mg/l) were 85.8–101.6% and 69.1–104.8%, respectively, and the limits of quantification for these insecticides were <0.01 mg/kg for grapes and <0.01 mg/l for wine. The method was applied to the determination of flufenoxuron and teflubenzuron residues in grapes from treated fields and in produced wine.

Keywords: Food analysis; Environmental analysis; Wine; Grapes; Sample preparation; Pesticides; Benzoylureas

1. Introduction

Wine, an important beverage in international trade, is subject to strict regulations concerning its quality in regards to truth-to-label and absence of additives [1]. Pesticides are used in vine protection and residues of them remaining on grapes at harvest may be transferred into the wine. Although pesticide residue levels are known to decrease during vinification [2,3], they are generally regulated through various national and international standards, established as maximum residue limits (MRLs), for pesticides in table or in viniferous grapes [4]. There is a world-wide trend towards setting specific, lower MRLs for pesticides in wine.

Among the different chemical classes of insecticides, which have been developed to control insect pests on vine, benzoylureas are promising, acting as powerful insect growth regulators [5]. Although their use is continuously increasing, due to their attractive properties, there are only a few analytical studies for their determination in some plant tissue matrices such as pear, apple, mushrooms and kiwi, but no

*Corresponding author. Tel: +30-421-742-42; fax: +30-421-633-83 or +30-421-635-44; e-mail: ntsirop@uth.gr

0021-9673/99/$ – see front matter
PII: S0021-9673(99)00053-9

analytical studies have appeared yet for their determination in grapes or in wine. Most of the analytical studies deal with the determination of a single benzoylurea, usually diflubenzuron [6–14]. Diflubenzuron is the only benzoylurea to be determined in different environmental samples both by gas chromatography (GC) [11–14], with or without derivatization, and by high-performance liquid chromatography (HPLC) [6–10]. The other ones are determined mainly by reversed-phase HPLC with UV detection [6,15]. Analytical studies concerned with the simultaneous determination of more than one benzoylurea in plant tissues, like apples and pears, have recently appeared in literature. Bicchi and co-workers [16,17] determined diflubenzuron, triflumuron and teflubenzuron in apple and pear pulp at 0.01 ppm level using extraction with dichloromethane–acetone, clean-up by C_{18} solid-phase extraction (SPE) and isocratic reversed-phase HPLC analysis with UV detection, or by supercritical fluid chromatography (SFC)–UV analysis after methanolic extraction and the same clean-up. Tomšej and Hajšlová [18] determined diflubenzuron, flufenoxuron, flucycloxuron, chlorfluazuron and triflumuron in apples using extraction with acetone, liquid partition into dichloromethane and gel permeation chromatography (GPC) clean-up before isocratic HPLC analysis on a C_8 column, with detection limits varying from 0.01 to 0.03 mg/kg for the different compounds.

Our laboratories have previously determined benzoylurea insecticides residues in apples and pears by using HPLC analysis after solvent extraction and polar SPE clean-up [19]. We now report the determination of diflubenzuron, triflumuron, teflubenzuron, lufenuron and flufenoxuron residues in grapes, after solvent extraction and silica SPE clean-up, and in wine, after the application of C_{18} SPE, in combination with narrow-bore gradient elution HPLC and UV diode array detection (DAD).

2. Experimental

2.1. Materials and reagents

Analytical standards of diflubenzuron (Solvay-Duphar, Weesp, The Netherlands), triflumuron (Bayer, Mannheim, Germany), lufenuron (Novartis, Basel, Switzerland) and flufenoxuron (Cyanamid, Princeton NJ, USA) were certified to be >99 % pure and teflubenzuron (Cyanamid) 97.4%. Ethyl acetate, *n*-hexane, dichloromethane and isopropanol were of pesticide residue grade; while water, methanol and ethanol were of HPLC grade; all solvents were purchased from Labscan (Dublin, Ireland).

2.2. Standard solutions

Individual analytical standard stock solutions of (a) 0.2 mg/ml for flufenoxuron, (b) 0.5 mg/ml for diflubenzuron and (c) 1 mg/ml for triflumuron, teflubenzuron and lufenuron were prepared in methanol and stored at −18°C in glass vials. A standard stock solution containing all compounds, at 100 μg/ml each, was prepared in methanol from the individual stock solutions. Standard solutions at concentrations 0.05 to 20.0 μg/ml were prepared from this standard stock solution to be used as calibration standards and spiking solutions.

2.3. Samples and recovery test

Grapes from untreated vines and white wine produced from them by microvinification were used as control samples, as well as for the fortification experiments. Commercial red and rosé wines were also used for the experiments.

Control grapes and wine samples were spiked at concentrations 0.02–2.0 mg/kg for grapes and 0.01–0.2 mg/l for wine. Commercial rosé and red wines were also spiked at 0.02 mg/l. Extraction of spiked samples were performed as described.

Grapes were also collected from vines treated, during summer 1997, with 0.08% (v/v) Nomolt 15SC (15%, w/v, teflubenzuron) and from vines treated with 0.075% (v/v) Cascade 10DC (10%, w/v, flufenoxuron). Grapes were collected from vines 40 and 50 days after treatment with Nomolt and Cascade, respectively. Wine was also produced from grapes collected from the treated vines.

All grape samples were homogenized and 50-g sub-samples were kept frozen until spiking or analysis.

2.4. HPLC apparatus and operation

Chromatographic analyses were performed with an HP 1090, Series II liquid chromatograph (Hewlett-Packard, Waldbronn, Germany), equipped with a ternary-delivery system attached to an autoinjector, a Model 1040 UV diode-array detector and a Chemstation chromatography manager data acquisition and processing system. The analytical column was an ODS Hypersil (MZ-Analysentechnik, Mainz, Austria) C_{18} column (250×2.1 mm I.D, 5.0 μm particle size).

The mobile phase was methanol–water, delivered at a flow-rate of 0.3 ml/min with a gradient composition, consisting of 10% (v/v) methanol for 5 min, a linear increase over 15 min to 64% methanol, then an increase to 100% methanol over 20 min and finally a decrease at 10% (v/v) methanol over 2 min, giving a total run time of 42 min. Acrodisk LC 13 (Gelman Sciences, Ann Arbor, MI, USA) PVDF membrane filters of 0.2 μm pore size were used for all samples. The injection volume was 20 μl and the column temperature 42°C. The optimum detection was at 260 nm, 4 nm bandwidth referenced against 450 nm wavelength, 80 nm bandwidth.

The concentration of the benzoylureas was determined by comparing the peak areas in the sample with those found in the calibration solutions. In addition to the spiked and treated samples, control samples were analyzed as blank samples and standard solutions were injected after every six samples. The calibration plots were reproduced weekly.

2.5. Sample preparation

2.5.1. Solvent extraction and clean-up for grapes

Solvent extraction of benzoylureas from grapes was based on a general extraction method using ethyl acetate as extraction solvent in the presence of anhydrous sodium sulphate [20]. To each homogenized sample (50 g) 100 ml ethyl acetate and 50 g sodium sulphate were added and the mixture was blended at low speed for 3 min. The supernatant extract was filtered through a Whatman No. 2 filter paper containing anhydrous sodium sulfate. Fifty ml of the filtrate was evaporated to dryness and the residue was redissolved, with the aid of an ultrasonic bath, in 5.0 ml hexane. Isolute SPE cartridges (International Sorbent Technology, Mid Glamorgan, UK), containing 500 mg of unbonded silica were used for the clean-up of samples. The cartridge was preconditioned with 10 ml hexane and after passage of the sample (1 ml) rinsed with 5 ml hexane. Benzoylureas were eluted with 2 ml dichloromethane–isopropanol (9:1). The eluent was taken to dryness under a gentle stream of nitrogen. Residues were redissolved in 1ml of methanol and the resulting solution was filtered prior to injection into the LC system.

2.5.2. Solid-phase extraction for wine

Wine (10 ml) was diluted with 30 ml of water and benzoylureas were extracted on a Isolute SPE cartridge containing 500 mg endcapped (EC) C_{18} sorbent. The C_{18} cartridge was preconditioned with 3 ml of acetone, 4 ml of 20% ethanol in water and finally with 4 ml of 2.5% ethanol in water. Diluted wine samples (40 ml) were passed through the cartridge under manual positive pressure with the aid of a glass syringe at a flow-rate of ca. 5 ml/min. After the enrichment step the cartridge was rinsed with 4 ml of 2.5% ethanol in water and the residues of this solvent were removed by passing 2×10 ml of air with the syringe, without drying the cartridge completely. Benzoylureas were eluted with methanol until collecting 2 ml of eluate. The extract was filtered and a suitable aliquot was injected into the LC system.

3. Results and discussion

Chromatographic analysis of the benzoylurea compounds was performed on octadecyl reversed-phase narrow-bore column, with gradient elution program providing adequate separation for the five compounds. The chromatograms of the grape and wine extracts were satisfactory, without interferences at the benzoylureas elution times area (Figs. 1 and 2).

For obtaining the calibration plots, calibration solutions of benzoylurea insecticides were injected in triplicate at the 1.0–100 ng range (eleven levels). The detector's response was linear over the studied range and the least-squares linear regression analysis of the data provided excellent correlation for all five compounds ($r>0.999$). The intercepts and the re-

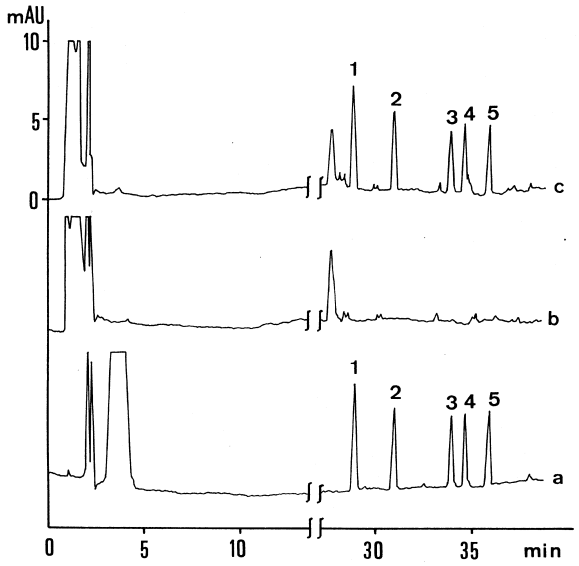

Fig. 1. Chromatograms obtained (a) for standard mixture of benzoylurea insecticides (10 ng) and after the solvent extraction and clean-up of (b) untreated grapes, (c) spiked grapes at concentration 0.1 mg/kg. 1=Diflubenzuron; 2=triflumuron; 3=teflubenzuron; 4=lufenuron; 5=flufenoxuron. Chromatographic conditions: see Section 2.4.

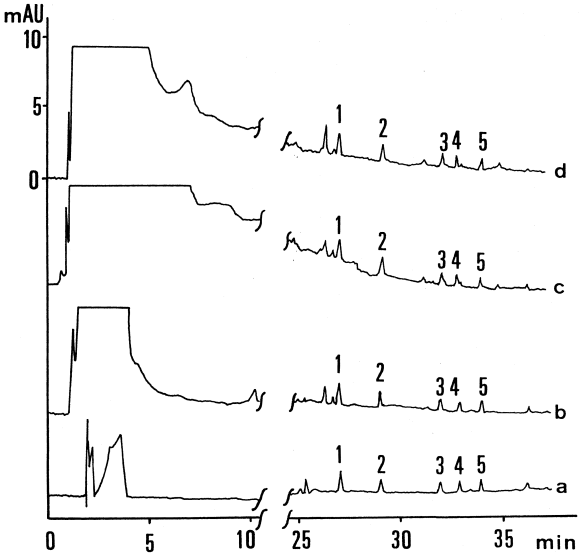

Fig. 2. Chromatograms of (a) standard mixture of benzoylurea insecticides (2 ng) and of spiked wines at 0.02 mg/l as follows: (b) white wine, (c) red wine and (d) rosé wine. Peak numbers as in Fig. 1. Chromatographic conditions: see Section 2.4.

sidual standard deviations for the five calibration curves were found to vary from −0.9 to 0.6 and from 0.8 to 2.6, respectively. Quantification of benzoylureas was performed by measuring the peak areas at 260 nm, a wavelength presenting high sensitivity with minor interferences.

3.1. Solvent extraction and clean-up of grapes

The proposed extraction by ethyl acetate–sodium sulfate is a general method for pesticides extraction from non fatty matrices, suited frequently for GC–nitrogen–phosphorus detection (NPD) analysis. By the use of this method benzoylurea compounds can be extracted in a frame of a multiresidue extraction method. In preliminary investigations performed for choosing the extraction solvent, this method presented the highest recoveries; other solvents tested were acetone and dichloromethane. The use of the polar silica stationary phase for benzoylureas clean-up was found efficient; these compounds were strongly retained by silica phase and elution was attained by the use of dichloromethane–isopropanol solution as mobile phase. The proposed sample preparation method is simple, fast and applicable to other matrices as well [19].

Accuracy and reproducibility of the described extraction, clean-up and analysis method for the five benzoylureas in grapes spiked at five levels were evaluated and are shown in Table 1. The mean recovery values at all concentrations levels were found to be in the range of 85.8 to 101.6% with relative standard deviation (RSD) values $\leq$5.8% for all compounds. The overall recovery for all spiking levels was 94.9% for diflubenzuron, 98.7% for triflumuron, 94.0% for teflubenzuron, 90.4% for lufenuron and 93.4% for flufenoxuron. All values in this table are within the accepted range for residue determinations [21].

3.2. Wine SPE and analysis

A simple SPE method for the isolation of benzoylurea insecticides from wine matrix was sought, as the use of SPE in pesticides analysis in wine is increasing and promising [22–26]. From the commercially available adsorbent phases the C_{18} bonded phase adsorbent was chosen, as it is widely used for

Table 1
Mean recovery (R) and relative standard deviation (RSD) (in parentheses) of benzoylurea insecticides from spiked grapes at various levels ($n=3$, except for spiking level 0.02 mg/kg, where $n=7$)

Pesticide	Mean recovery (R, %) (RSD, %)				
	0.02 mg/kg	0.1 mg/kg	0.5 mg/kg	1.0 mg/kg	2.0 mg/kg
Diflubenzuron	87.4 (4.5)	95.8 (2.1)	97.2 (2.5)	96.8 (1.0)	97.3 (1.4)
Triflumuron	100.1 (2.7)	100.8 (1.4)	101.6 (2.2)	92.9 (0.9)	97.9 (4.2)
Teflubenzuron	87.1 (3.2)	87.7 (2.0)	96.2 (0.5)	99.7 (0.5)	99.4 (0.8)
Lufenuron	90.8 (5.8)	90.4 (1.9)	97.4 (0.8)	87.6 (1.5)	85.8 (3.5)
Flufenoxuron	98.9 (2.4)	93.6 (2.5)	99.1 (0.8)	87.5 (0.7)	87.7 (2.2)

pesticide determination or screening in wine samples [25,26]. The 2 ml of elution solvent (methanol) used was found adequate for extracting the benzoylureas, as they were not detected in the eluent resulting by passing an additional 2 ml of elution solvent through the cartridge. After SPE tests of benzoylurea pesticides from spiked undiluted wine samples, satisfactory recovery values (>91.6%) at all concentrations tested (0.02, 0.1 and 0.2 mg/l) were observed only for diflubenzuron and triflumuron, while low or insufficient values were observed for teflubenzuron (54–95%), lufenuron (47–67%) and flufenoxuron (52–75%), particularly at the spiking levels 0.1 and 0.2 mg/l (Fig. 3). Recovery was found to improve when wine diluted with water was spiked and extracted. The highest recoveries (>69.1% at all spiking levels) were found after wine dilution with water at 1:3 ratio, while the recovery values after a dilution at 1:1 and 1:2 were >59.5% and >63.2%, respectively. Recoveries were also found to be dependent on the concentration of the benzoylurea insecticides (mainly for flufenoxuron and lufenuron and less for teflubenzuron) in wine, i.e., decreased with increasing concentration (Fig. 3 and Table 2). Although this dependence was rather unimportant in the case of diflubenzuron and triflumuron, it was important for consideration in the case of flufenoxuron and lufenuron, especially at the highest concentrations (0.1 and 0.2 mg/l). Nevertheless, the method remains simple and rapid, and achieves satisfactory accuracy and precision for residue analysis, as shown in Table 2, where the results of the validation of the proposed extraction procedure, by spiking wine samples at five levels, are given. The mean recovery values were between 69.1 to 104.8% for all insecticides at all spiking levels. The recoveries were above 87% for diflubenzuron, triflumuron and teflubenzuron at all concentrations tested and for flufenoxuron and lufenuron at concentrations 0.01, 0.02 and 0.05 mg/l, while at higher concentrations (0.1 and 0.2 mg/l) the recoveries for these two insecticides ranged between 69.1 and 77.5%. Reproducibility was also very good for all pesticides, as the RSD ranged from 0.8 to 5.8%.

The extraction procedure for benzoylurea insecticides was also evaluated by spiking Greek wine of two varieties: red and rosé. The results are showed in Table 3 and the relative chromatograms are presented in Figs. 2c and d. The recoveries found (73–110%) were in the same range as for white wine, while a small dependence on the variety of wine was observed, showing a minor influence of the wine matrix on the extraction process.

3.3. Limits of quantification

The limits of quantification (LOQs), evaluated as signal-to-noise (S/N) ratio equal to 10, with the selected gradient elution program and wavelength detection, were found to be 0.4 to 0.5 ng for the five insecticides. These values, combined with the proposed sample preparation and chromatographic injection volume (20 μl), correspond to LOQs of 0.004–0.005 mg/kg in grapes and 0.004–0.005 mg/l in wine and enable reliable determinations of benzoylureas insecticides at least at the 0.01 ppm level.

If lower concentrations need to be determined (especially in wine) it can possibly be attained by either concentrating the final methanolic solution before LC injection or by injecting a larger volume (30 instead of 20 μl). Injection volumes in excess of 30 μl resulted in peak broadening, due to a dilution effect.

The LOQ values are comparable to those pub-

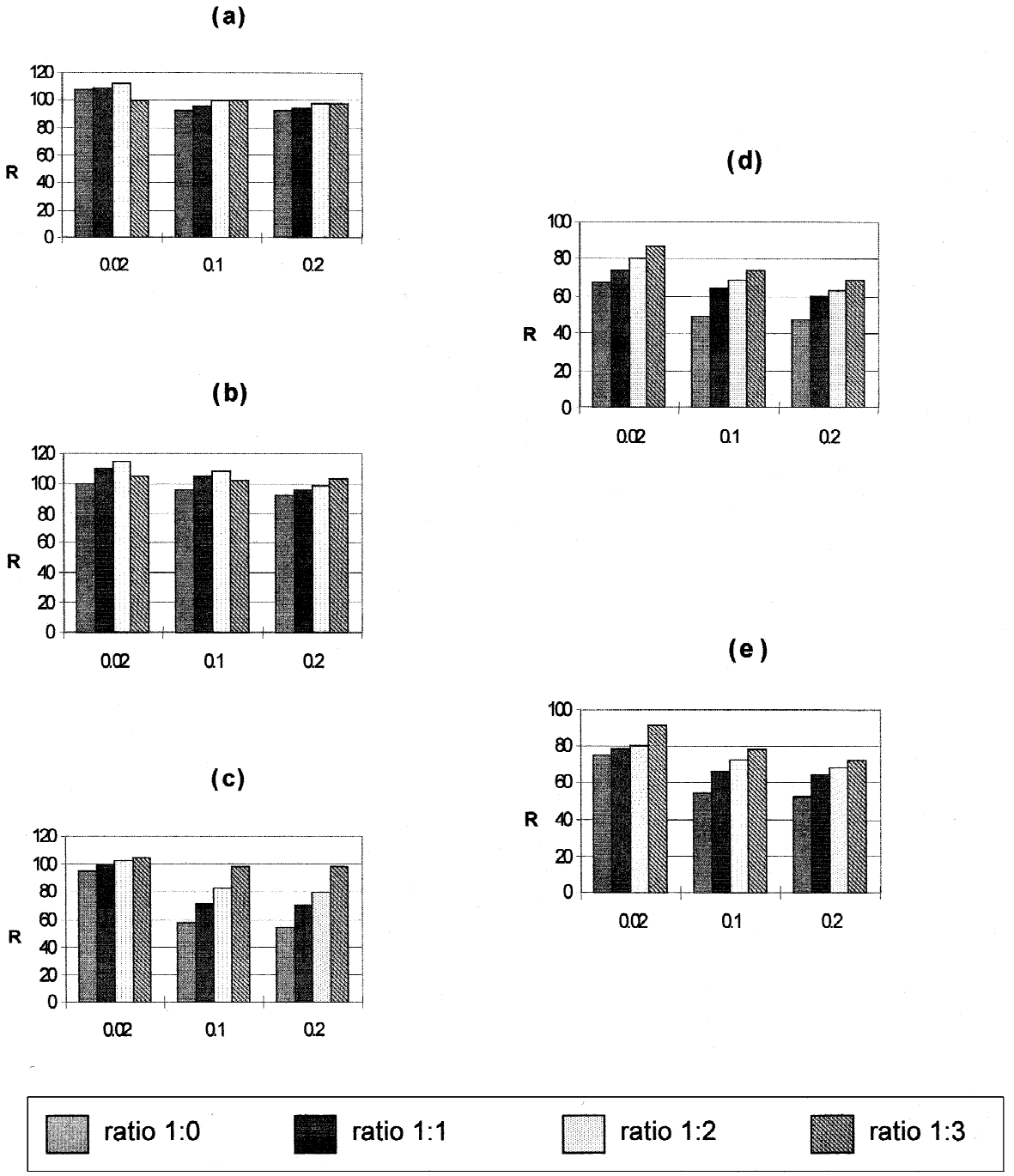

Fig. 3. Recoveries (%) (*R*) of benzoylurea insecticides after solid-phase extraction from spiked wine at various levels (0.02, 0.1 and 0.2 mg/l) and at different dilution wine:water ratios (1:0, 1:1, 1:2, 1:3). (a), Diflubenzuron; (b), triflumuron; (c), teflubenzuron; (d), lufenuron; (e), flufenoxuron.

Table 2
Mean recovery (*R*) and relative standard deviation (RSD) (in parentheses) of benzoylurea insecticides from spiked wine at various levels ($n=4$, except for spiking levels 0.01 and 0.02 mg/kg, where $n=7$)

Pesticide	Mean recovery (*R*, %) (RSD, %)				
	0.01 mg/l	0.02 mg/l	0.05 mg/l	0.1 mg/l	0.2 mg/l
Diflubenzuron	100.6 (2.6)	99.2 (2.6)	94.4 (4.7)	99.2 (2.6)	97.4 (2.5)
Triflumuron	98.3 (3.0)	104.8 (3.6)	98.3 (4.3)	101.4 (1.7)	102.7 (0.8)
Teflubenzuron	101.8 (4.0)	104.7 (3.9)	97.9 (3.1)	97.6 (2.8)	98.2 (5.4)
Lufenuron	97.1 (2.9)	87.0 (3.0)	90.8 (4.7)	73.5 (1.2)	69.1 (2.9)
Flufenoxuron	99.2 (3.6)	90.9 (3.2)	91.7 (1.7)	77.5 (3.0)	72.1 (5.8)

lished for benzoylurea analysis in various plant tissues [10,11,16–19]. No MRLs have been set by the European Union or the FAO/WHO for the five benzoylureas in grapes. Various countries have set MRLs, e.g., Italy has set 1 mg/kg for teflubenzuron, 0.5 mg/kg for lufenuron and 0.1 mg/kg for flufenoxuron in grapes; France has set 0.1 mg/kg for flufenoxuron in grapes. The LOQs attained by the method are much lower than those MRL values, making the method suitable for routine analysis.

3.4. Field sample analysis

Residues of teflubenzuron and flufenoxuron measured on field treated grapes and on wine produced from them, as described in Section 2.3, were found to be 0.52 and 0.20 mg/kg for the two compounds respectively in grapes, and 0.012 and 0.010 mg/l, respectively in wine. By comparing these values, it can be seen that concentrations of teflubenzuron and flufenoxuron residues are considerably decreased from vine to wine. The determined concentrations are within the studied range and the application of the method was proved satisfactory.

Table 3
Mean recovery (*R*) and relative standard deviation (RSD) (in parentheses) of benzoylurea insecticides, as evaluated from wines of different varieties (white, rosé, red) at 0.02 mg/l ($n=4$, except for white wine, where $n=7$)

Pesticide	Mean recovery (*R*, %) (RSD, %)		
	White	Rosé	Red
Diflubenzuron	99.2 (2.6)	100.1 (2.8)	103.5 (5.4)
Triflumuron	104.8 (3.6)	110.0 (4.1)	109.7 (4.7)
Teflubenzuron	104.7 (3.9)	87.3 (5.2)	91.8 (7.2)
Lufenuron	87.0 (3.0)	84.3 (4.9)	82.1 (6.1)
Flufenoxuron	90.9 (3.2)	73.0 (4.6)	74.3 (3.7)

4. Conclusions

A novel and robust method for the determination of benzoylurea insecticides residues in grapes and wine was developed. Reliable recovery data were found at various concentrations, after spiking grapes and wine, and good limits of quantification were attained. Solvent extraction and SPE clean-up for grapes, and SPE for wine were used in combination with narrow-bore HPLC with UV–DAD under optimized experimental conditions. The method is suitable for routine analysis for grapes and wine, as well as for research dissipation studies of these insecticides in field-treated grapes.

References

[1] S. Brun, J.A. Cabanis, J.P. Mestres, Experientia 42 (1986) 892.
[2] C. Sala, F. Fort, O. Busto, F. Zamora, L. Arola, J. Guasch, J. Agric. Food Chem. 44 (1996) 3668.
[3] P. Cabras, M. Meloni, F.M. Pirisi, Rev. Environ. Contam. Toxicol. 99 (1987) 83.
[4] Compte Rendu de la Sous Commission Conventionnelle d'Unification des Methodes d'Analyse et d'Appreciation des Vins (35th session), Bull. OIV, 763–764 (1994) 786.
[5] C. Tomlin, The Pesticide Manual, 10th ed, British Crop Protection Council and The Royal Society of Chemistry, London, 1994.
[6] K.M.S. Sundaram, R. Nott, J. Liq. Chromatogr. 12 (1989) 2333.
[7] D.J. Austin, K.J. Hall, Pestic. Sci. 12 (1981) 495.
[8] S.M. Walters, B.C. Westerby, D.M. Gilvidys, J. Chromatogr. 317 (1984) 533–544.
[9] S.J.D. Prima, R.D. Cannizzaro, J.C. Roger, C.D. Ferrelli, J. Agric. Food Chem. 26 (1978) 968.
[10] K.A. Barnes, J.R. Startin, S.A. Thorpe, S.L. Reynolds, R.J. Fussell, J. Chromatogr. A 712 (1995) 85.
[11] J.K. Mensah, E. Lundanes, T. Greibrokk, B. Holen, J. Chromatogr. A 765 (1997) 85.

[12] H.J. Stan, P. Klaffenbach, Fresenius J. Anal. Chem. 339 (1991) 40.
[13] H.N. Nigg, R.D. Cannizzaro, J.H. Stamper, Bull. Environ. Contam. Toxicol. 36 (1986) 833.
[14] M.J. Wimmer, R.R. Smith, J.P. Jones, J. Agric. Food Chem. 39 (1991) 280.
[15] W.A. Hopkins, D.R. Lauren, J. Chromatogr. 516 (1990) 442.
[16] C. Bicchi, C. Balbo, A. Binello, A. D'Amato, J. High Resolut. Chromatogr. 19 (1996) 105.
[17] C. Bicchi, C. Balbo, A. D'Amato, O. Panero, Chromatographia 43 (1996) 439.
[18] T. Tomšej, J. Hajšlová, J. Chromatogr. A 704 (1995) 513.
[19] N.G. Tsiropoulos, P.G. Aplada-Sarlis, G.E. Miliadis, J. Assoc. Off. Anal. Chem. 82 (1999) 213.
[20] Analytical Methods For Pesticide Residues in Foodstuffs, 6th ed, Ministry of Public Health, Welfare and Sport, Bilthoven, 1996, Part I, p. 4.
[21] Council Directive 94/43/EC Off. J. Eur. Com. L227, 1994, pp. 31–55
[22] E. Matisova, L. Kakakilova, J. Lesko, J. de Zeeuw, J. Chromatogr. A 754 (1996) 445.
[23] P. Cabras, C. Tuberoso, M. Melis, M.G. Martini, J. Agric. Food Chem. 10 (1992) 817.
[24] J. Simal, P. Paseiro, V. Gonzalez, A. Romero, J. Agric. Food Chem. 41 (1992) 674.
[25] P.T. Holland, D.E. McNaughton, C.P. Malcolm, J. Assoc. Off. Anal. Chem. 77 (1994) 79.
[26] A. Caufman, J. Assoc. Off. Anal. Chem. 80 (1997) 1302.