Application of an ELISA to the Determination of Benalaxyl in Red Wines

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The applicability of an ELISA for detection and quantification of benalaxyl in red wine samples is described. The study of the influence of this matrix on the reliability of the assay indicates that red wine samples require a rapid and simple cleanup step before ELISA assay. Recovery and precision of the method were evaluated by spiking red wine samples with benalaxyl in the 0.5−24 ng/mL range. Benalaxyl can be determined with good accuracy and precision up to 0.5 ng/mL in starting red wine samples (detection limit of 0.13 ng/mL). No false negative or positive results were obtained. Authentic red wine samples were analyzed by ELISA and by RP-HPLC. The amounts of benalaxyl found by ELISA were in good agreement with RP-HPLC analysis.

Keywords: *Benalaxyl; enzyme-linked immunoassay; analysis; red wine*

INTRODUCTION

Benalaxyl (methyl *N*-phenylacetyl-*N*-2,6-xylyl alaninate) is a xylem-systemic fungicide extensively used on grapes, tomatoes, peppers, potatoes, onions, and tobacco because of its effectiveness in inhibiting pathogen growth within the plant tissue and in inhibiting *Plasmopara viticola zoospore* germination on the surface of the plant (Gozzo et al., 1985). Benalaxyl is determined in routine food analysis; the present European legislative purpose requires a limit of 0.2 mg/L for benalaxyl on grapes, peppers, and tomatoes and a limit of 0.05 mg/L on other vegetables (Gazzetta Ufficiale, 1998). Benalaxyl acute oral LD_{50} in laboratory mice is 680 mg/kg (Tomlin, 1994).

Instrumental methods usually employed for benalaxyl analysis—mainly RP-HPLC (Cabras et al., 1987) or GC−NPD (Crisippi et al., 1993)—work well but are time-consuming and involve laborious extraction and cleanup procedures. Competitive enzyme-linked immunosorbent assays (ELISAs) are becoming either alternative or complementary analytical tools to conventional methods because of their desirable analytical features such as rapidity, sensitivity, selectivity, and low cost. In practice, one of the most important advantages of immunoassays for pesticide analysis lies in their minimal requirement of sample cleanup (Brecht et al., 1995).

Up to now many immunoassays for pesticides have been developed (Hock, 1995). Most of these have been applied to water analysis (Hermion et al., 1998) but a lower number to more complex matrixes (Nunes et al., 1998; Mercader et al., 1997). On the other hand, only one immunoassay to an acylalanine fungicide (metalaxyl) has been described (Newsome, 1985). Recently, the development of a specific and sensitive ELISA for benalaxyl in aqueous samples and its application for the analysis of white wines have been reported (Giraudi et al., 1999). In the present work, the suitability of this ELISA for the analysis of benalaxyl residues in red wines was evaluated. To this purpose, matrix effects on the reliability of the assay were studied; the ELISA was subsequently applied to spiked and authentic red wine samples that were also analyzed by RP-HPLC.

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MATERIALS AND METHODS

Chemicals and Instruments. Benalaxyl with a purity of 99.9% was from Dr. Ehrenstorfer (Augsburg, Germany). Stock solutions of the pesticide were prepared in methanol at 10 mg/L concentration and stored at 4 °C. The specific polyclonal chicken antiserum to benalaxyl was produced from the hapten 4-[2-(*N*-phenylacetyl-*N*-2,6-xylylamino)propionamido]butyric acid (Bn-AB) as previously described (Giraudi et al., 1999). The methyl *N*-phenylacetyl-*N*-2,6-xylyl alanine (Bn-COOH)−OVA conjugate (reaction ratio of 40:1) was prepared as previously described (Giraudi et al., 1999). Peroxidase-labeled goat anti-chicken γ-globulins and bovine serum albumin (BSA) were from Sigma (Milwaukee, WI). Chromogen, tetramethylbenzidine, and substrate, hydrogen peroxide, reagents for HRP in citrate buffer were supplied by Sorin Biomedica (Saluggia, Italy) and were ready to use. All chemicals for buffers were from Merck (Darmstadt, Germany).

Polystyrene microtiter plates (8 × 12 wells) were from Nunc (Roskilde, Denmark). Extract Clean C-18 tubes (200 mg) were from Alltech Italia (Sedriano, Italy).

Microtiter plate washer (Novapath washer), incubator (Microplate incubator), and reader (Microplate Reader 3550) were from Bio-Rad (Hercules, CA).

A Hewlett-Packard HP 1100 high-pressure chromatographic apparatus equipped with an 1100 binary pump and with a UV−vis/diode array detector (HP 1100 DAD, Woldbronn, Germany) was employed. A Merck column RP-selected B, 5 μm, 250 mm × 3 mm, was used.

Sample Preparation. A red wine sample (Dolcetto 1) was collected from vineyards in the vicinity of Cuneo (Italy) where benalaxyl was not applied; other samples (Barbera, Grignolino) were kindly provided by G. Bosca, Coldiretti Asti (Italy), from vineyards in which benalaxyl was applied. The other red wines (Dolcetto 2, Lambrusco, Brachetto, Nebbiolo) are commercial samples obtained from local supermarkets.

Wines were spiked with benalaxyl from a solution of 100 μg/mL in methanol to reach a final concentration between 0 and 50 ng/mL, before any pretreatment.

A tannin precipitation method drawn from the literature was applied to sample Dolcetto 1 (Makkar, 1989): 1 mL of

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sample was added to 2 mL of acetate buffer (0.2 M acetate, 0.17 M NaCl, 0.1% w/v BSA, pH 5). After 15 min of stirring and 30 min of centrifugation, the supernatant was analyzed by ELISA.

To achieve a more complete removal of tannins, an optimized cleanup step was performed on all of the samples: 1.5 mL of each sample was passed through to a SPE C-18 minicolumn, previously equilibrated with 3 mL of methanol. The column was eluted with 1 mL of 50% ethyl ether in hexane and, after evaporation to dryness, the residue was dissolved in 12% ethanol−PBS buffer (20 mM phosphate, 0.13 M NaCl, 1 mM EDTA, 12% v/v ethanol, pH 7.4) for immunoassay and chromatographic analysis.

Benalaxyl Immunoassay. A competitive indirect ELISA format was used. Incubation times, coating conjugate, and antibody concentrations were optimized as described in the preceding paper (Giraudi et al., 1999). Working dilutions of benalaxyl standard solution were prepared by diluting stock solution with 12% ethanol−PBS buffer in the range from 0.3 to 300 ng/mL. The calibration curve and the analysis of samples, treated as above-described, were set up on Bn-COOH−OVA coating antigen, immobilized at 1 μg/mL concentration in 96-well microtiter plates. Wells were blocked as previously described (Giraudi et al., 1999), adding 2% w/v PVP as stabilizer. Chicken antiserum to benalaxyl was used at 1:5000 dilution in PBS−BSA buffer (0.1 M phosphate, 0.05 M NaCl, 1 mM EDTA, 1% w/v BSA, pH 7.4).

One hundred microliters of the standard or of the sample solution and 100 μL of diluted antiserum were added in duplicate for the standard and six times for the sample solution to each well. Nonspecific binding was measured by substituting diluted antiserum with buffer. Plates were left to incubate overnight at room temperature and washed three times with a washing solution (0.05% v/v Tween 20). Then, 200 μL of the enzyme-labeled antibody, diluted 1:4000 into PBS−gelatin buffer (20 mM phosphate, 0.13 M NaCl, 1 mM EDTA, 0.1% w/v gelatin, 0.05% v/v Tween 20, pH 7.4) was added to each well. Plates were incubated for 1 h at 37 °C and washed three times.

The colorimetric reaction was performed by adding 200 μL of chromogen/substrate solution (1+1) to each well. After 15 min of incubation at 37 °C in the dark, enzymatic reaction was blocked by adding 100 μL of 1 M sulfuric acid; the absorbance was then read at 450 nm.

Calibration curves (absorbance at 450 nm versus benalaxyl concentration) were fitted using the four-parameter logistic equation of Rodbard (IUPAC, 1995). Benalaxyl concentration of samples was determined by interpolation of the absorbance values on the calibration curve.

Chromatographic Analysis. After the cleanup step, the samples were analyzed by HPLC, mobile phase water/acetonitrile (45:55% v/v), flow rate = 1 mL/min, injection volume = 20 μL. The absorbance of analyte was monitored at 200 and 230 nm. External standard calibration graph was obtained by using benalaxyl standard solution in methanol. Linear range was from 10 to 300 ng/mL, and the detection limit of benalaxyl was 10 ng/mL (according to a signal/noise ratio equal to 2).

RESULTS AND DISCUSSION

Analytical Characteristics of the ELISA for Benalaxyl. Eight standard curves prepared in 12% ethanol−PBS buffer for the benalaxyl competitive immunoassay are shown in Figure 1.

The detection limit, defined as the concentration of benalaxyl equivalent to three standard deviations in observations at B_0 (the binding measured in the absence of benalaxyl), is 0.13 ng/mL. The midpoint value (I_{50}), evaluated as the concentration of benalaxyl at 50% B/B_0, is 2.0 ng/mL. The working range, evaluated as the benalaxyl concentration that gives test inhibition values of 90% of $\%B/B_0$ (I_{90}) and 10% of $\%B/B_0$ (IUPAC, 1995) is between 0.4 and 40 ng/mL.

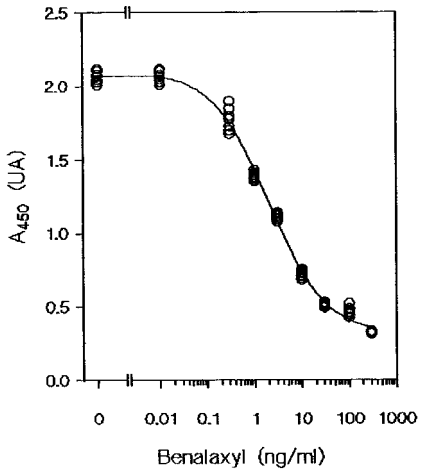

Figure 1. Intra-assay variation (eight standards) for benalaxyl calibration curve. The standard curve (prepared in 12% ethanol−PBS buffer) was obtained on Bn-COOH−OVA (40:1) solid phase immobilized at 1 mg L^{-1} concentration, using 1:5000 diluted chicken antiserum.

Study of Matrix Effects. Immunoassay performance is usually influenced by food matrixes. Therefore, matrix effects should be determined before an assay can be applied to samples containing the pesticide. The easiest and most immediate way to minimize matrix effects is sample dilution. A sample dilution 1+3 in 12%−ethanol PBS buffer is required to determine benalaxyl quantitatively in white wines samples (Giraudi et al., 1999). Regarding red wines—the matrixes selected for this work—a more complex study is necessary. A red wine sample (Dolcetto1), spiked with benalaxyl at 2, 5, 20, 50, and 200 ng/mL, was directly analyzed by ELISA without any pretreatment at all or diluted 1+1, 1+3, and 1+7 in 12% ethanol−PBS buffer. Recoveries completely not quantitative—even from the more diluted sample—are obtained, probably because antibody molecules are involved in nonspecific interactions with tannins and dyes of red wine.

To remove tannins, the precipitation method described under Materials and Methods was applied to Dolcetto 1. Standard curves prepared in 12% ethanol−PBS buffer and in the precipitation buffer (0.2 M acetate, 0.17 M NaCl, 0.1% w/v BSA, pH 5) are shown in Figure 2. The acetate buffer standard curve cannot be used as a calibration curve because of its limited slope and working range. On the other hand, a quantitative recovery of benalaxyl from red wine samples—after the precipitation step—on the PBS buffer calibration curve cannot be obtained, as reported in Table 1 for Dolcetto 1 spiked with benalaxyl before the precipitation.

Acetate buffer composition was varied to increase the slope and the working range of the corresponding standard curve. As shown in Figure 2, the standard curve prepared in 0.1 M acetate buffer, pH 5, offers a larger working range. However, the recovery of 0.5, 5, and 50 ng/mL of benalaxyl spiked into Dolcetto 1 either on the PBS buffer calibration curve or on the 0.1 M acetate buffer calibration curve, when 0.1 M acetate buffer is employed in the precipitation step, is not quantitative in a large working range yet (Table 1).

The neutralization of the supernatant by a small amount of diluted NaOH solution causes obtained absorbance values so high that a recovery evaluation is not possible. Also, the increase of buffer capacity of

Table 1. Recovery of Benalaxyl in Dolcetto 1 Sample (14% vol) on Different Buffer Calibration Curves When a Tannin Precipitation Step Was Applied on the Sample Using Different Precipitation Buffers

precipitation buffer	buffer calibration curve	benalaxyl added (ng/mL)	recovery (%) of undiluted supernatant	recovery (%) of supernatant diluted 1+1
0.2 M acetate, pH 5	12% ethanol−PBS buffer	0.5	174	
		5	95	97
		50	12	15
0.1 M acetate, pH 5	12% ethanol−PBS buffer	0.5	288	
		5	110	104
		50	17.5	16
0.1 M acetate, pH 5	12% ethanol−0.1 M acetate, pH 5	0.5	299	
		5	127	115
		50	16.8	14

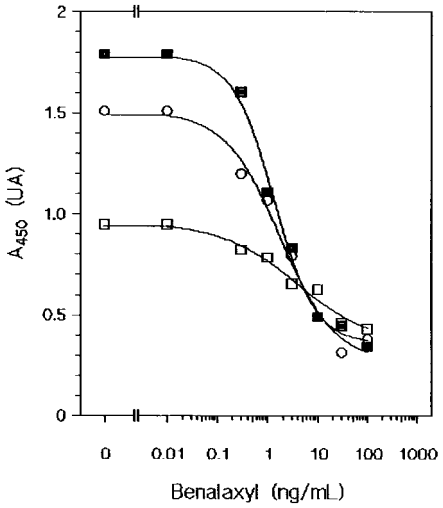

Figure 2. Benalaxyl calibration curves prepared in (■) 12% ethanol−PBS buffer, in (□) 12% ethanol−0.2 M acetate buffer (0.2 M acetate, 0.17 M NaCl, 0.1% w/v BSA, pH 5), and in (○) 12% ethanol−0.1 M acetate buffer (0.1 M acetate, 0.17 M NaCl, 0.1% w/v BSA, pH 5).

Table 2. Recovery of Benalaxyl in Dolcetto 1 Sample (14% vol) on a Calibration Curve Prepared in 12% Ethanol−PBS Buffer or 12% Ethanol−2% PVP−PBS Buffer (*) When the Composition of Antibody Dilution Buffer or Precipitation Buffer Is Modified

modification	benalaxyl added (ng/mL)	recovery (%) of undiluted supernatant	recovery (%) of supernatant diluted 1+1
antibody dilution buffer:	0.5	91	112
0.2 M PBS−BSA	5	23	21
	50	9.6	
precipitation buffer:	0.5	424*	194*
2% PVP−0.1 M	50	254*	97*
acetate	150	355*	269*

antibody dilution buffer gives unsatisfactory results, as reported in Table 2.

The precipitation step allows us to clarify red wine samples but not to remove all of the dyes. As dyes are involved in nonspecific interactions with various reagents, it was evaluated if matrix effects were linked to PVP presence, as this reagent was used during the coating procedure. Standard curves prepared in 12% ethanol−PBS buffer with different PVP percentages (0, 0.1, 0.5, 1, and 2% w/v PVP) show that the maximum response (B_0) does not vary significantly, whereas the slope and the working range are higher when 2% PVP−12% ethanol−PBS buffer is used. Dolcetto 1 samples, spiked with benalaxyl, were treated by the precipitation step employing 2% PVP−0.1 M acetate buffer. The recovery of benalaxyl on the calibration curve prepared in 2% PVP−12% ethanol−PBS buffer is still completely not quantitative (Table 2).

Table 3. Recovery of Benalaxyl in Dolcetto 1 Sample (14% vol) on a Calibration Curve Prepared in 12% Ethanol−PBS Buffer When a Cleanup Step on C-18 SPE Columns Was Applied to the Sample

benalaxyl added (ng/mL)	amount expected[a] (ng/mL)	amount determined[b] (ng/mL)	recovery (%)
0	0	0	100
0.5	1.5	1.8	123
1.4	4.2	4.3	103
5	15	15.6	104
14	42	41.6	99

[a] Considering concentration factor. [b] The amount determined is the average of six value readings with a coefficient of variation varying from 8 to 20% in all of the considered range.

To reduce matrix interferences drastically, a cleanup step is necessary, so samples were passed through to C-18 SPE minicolumns. The procedure described under Materials and Methods is the result of preliminary experiments in which the column loading and the optimum eluent volume were evaluated to obtain quantitative recovery of benalaxyl with 0.5 mL of 12% ethanol−PBS buffer. The recovery of 0, 0.5, 1.4, 5, and 14 ng/mL of benalaxyl spiked into Dolcetto 1 sample—before the cleanup step—on the 12% ethanol−PBS buffer calibration curve is reported in Table 3. Quantitative recoveries ranging from 99 to 123% in a working range of ∼1.5−40 ng/mL result. Considering the concentration factor equal to 3, benalaxyl can be determined up to 0.5 ng/mL in starting red wine samples.

Determination of Benalaxyl in Authentic Red Wine Samples. Six different kinds of red wine samples, of alcoholic strength varying from about 10 to 14% volume, were analyzed using standard addition method by ELISA. Each sample was spiked with benalaxyl at 1, 3, 6, 12, or 24 ng/mL concentration, before the cleanup step. Moreover, an amount of nonspiked sample was directly cleaned up and then analyzed by ELISA. Each ELISA plate included its own benalaxyl standard curve in 12% ethanol−PBS buffer, and absorbances from samples were interpolated on the curve performed in the same plate. Linear regression analysis indicates a good correlation between benalaxyl concentration added to red wine samples and benalaxyl concentration determined by ELISA. The intercept of the straight-line curve indicates the concentration of the fungicide eventually present in each sample, and it can be compared with the result of ELISA analysis on nonspiked samples. Table 4 reports the parameters of linear regressions on average values (n = 6), obtained for each analyzed sample, and the mean of six ELISA determinations on nonspiked samples. The good agreement between the

Table 4. Parameters of Linear Regressions on Average Values ($n = 6$), Obtained for Each Sample Analyzed by Standard Addition Method, and Comparison to the Mean of Six ELISA Determinations on Nonspiked Samples

	standard addition method			direct analysis
sample	slope	intercept (ng/mL)	correl coeff	amount determined[a] (ng/mL)
Dolcetto 2, 14% vol	1.04	11.6	0.99	12.1 ± 0.5
Barbera, 12.5% vol	1.08	6.4	0.99	6.0 ± 0.6
Nebbiolo, 12% vol	1.01	3.4	0.99	3.6 ± 0.2
Grignolino, 12% vol	1.12	1.7	0.99	1.5 ± 0.3
Brachetto, 11% vol	1.02	4.5	0.99	4.5 ± 0.4
Lambrusco, 10% vol	0.89	0.01	0.99	0.4 ± 0.1

[a] Mean ± standard deviation.

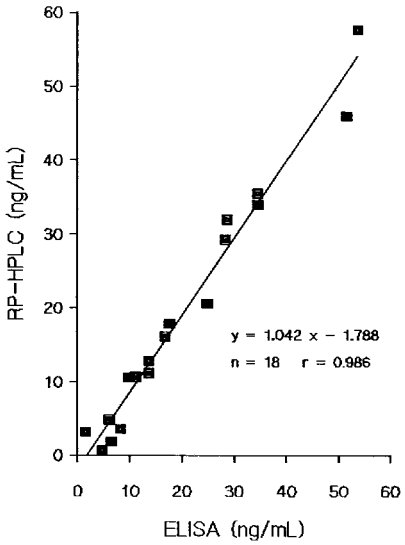

Figure 3. Correlation of RP-HPLC versus ELISA results for red wine samples spiked with benalaxyl.

benalaxyl concentration determined by the standard addition method and the benalaxyl concentration determined by ELISA direct analysis attests to the reliability of both the cleanup step and the ELISA developed for the determination of benalaxyl in red wine. It is important to emphasize that ELISA direct analysis is better than the standard addition method because it provides a more precise measurement.

Data obtained by recovery experiments on spiked and unspiked samples show that the coefficient of variation on a single sample varies from 4 to 10% in a range between ~3 and ~15 ng/mL and from 10 to 20% of the first considered range.

To confirm the reliability of ELISA analysis, some of the same spiked samples were analyzed also by RP-HPLC after the cleanup step. Correlation of RP-HPLC versus ELISA results is reported in Figure 3. It is evident that ELISA yields comparable values and reliable information about the degree of contamination in wine samples. The worst agreement between the two techniques, at a benalaxyl concentration <10 ng/mL, is attributable to the imprecision of HPLC determination at concentrations lower than the sensitivity of the chromatographic technique. The most remarkable aspect is the good detection of benalaxyl residues by ELISA at very low levels (0.5 ng/mL), whereas at this concentration no detection was achieved by RP-HPLC. Although ELISAs are generally less precise than chromatographic methods, they represent a feasible alternative to conventional analytical techniques for determination of agrochemical residues in food supplies, thanks to their higher sensitivity, rapidity, and lower expenses.

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