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Gas chromatographic ion trap mass spectrometry determination of zoxamide residues in grape, grape processing, and in the fermentation process

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Abstract

A gas chromatographic ion-trap mass spectrometry (GC-ITMS) method was developed for the determination of the fungicide zoxamide in grape, must, wine, and spirits. Samples were extracted with hexane and analyzed without any clean up. The gas chromatograph was fitted with a carbofrit inserted into the glass liner to allow large volume injections. Analyses were carried out both in EI and CI mode. Recoveries from fortified samples ranged between 86 and 114% at four different fortification levels ($n = 6$ each), ranging between 0.05 and 2.00 mg/kg. The relative standard deviation was below 19%. Both in EI and CI mode the calculated limit of detection (LOD) and quantification (LOQ) were 0.01 and 0.05 mg/kg (0.08 mg/kg in CI), respectively. Moreover the influence of yeasts and bacteria fermentation was evaluated.

Keywords: Zoxamide; Ion trap mass spectrometry; Food analysis; Grape; Must; Wine; Spirits; Yeasts; Bacteria

1. Introduction

Downy mildew induced by *Plasmopara viticola* is a major illness that affects wild and cultivated grapes (*Vitis species*). This fungus is an obligate biotrophic parasite, and it occurs throughout all-important wine growing regions in temperate climates. Downy mildew is controlled by methodology, which combine breeding and chemical methods. In order to achieve an effective control of this fungus it is necessary to use fungicides at the right stage of growing [1]. The best period is immediately before flowering. Among the chemicals currently being used to control this pest, zoxamide (Fig. 1), (*RS*)-3,5-dichloro-*N*-(3-chloro-1-ethyl-1-methyl-2-oxopropyl)-4-methyl-benzamide, is a new fungicide belonging to the benzamide family [1,2] for foliar use on potatoes, vines, and vegetables. It exhibits a strong preventive activity combined with excellent rainfast and residual properties. This fungicide acts after spore germination to arrest germ tube elongation, to control mycelia growth, and inhibits fungal penetration [3–5]. To our knowledge only data from the producer are reported concerning analytical methods for the analysis of this fungicide in grape, must, wine and spirits. The aim of this paper is to develop a simple and rapid method, which allows the determination of zoxamide in grapes, wine, and their distilled products (brandy and grappa).

2. Experimental

2.1. Chemicals

Zoxamide was an analytical standards kindly donated by the manufacturer (Dow AgroScience) at 99% certified purity. Acetone and hexane were residue analysis grade, purchased from Carlo Erba (Milan, Italy). Sodium metabisulfite, sodium chloride and anhydrous sodium sulfate were of analytical grade

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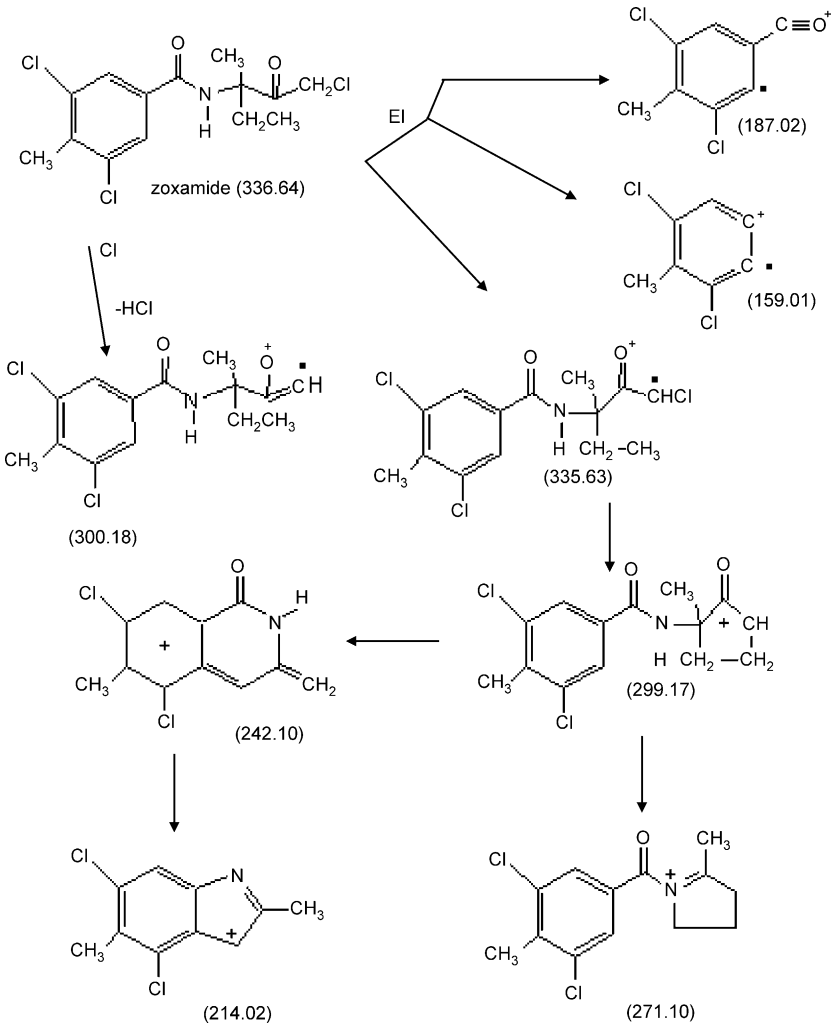

Fig. 1. Zoxamide and its degradation pathway in GC-ITMS analysis under EI and CI mode.

(Carlo Erba, Milan, Italy). Stock standard solution of zoxamide was prepared dissolving 50 mg of analytical standard in 10 mL of acetone and was stored in glass screw capped flask at −20 °C. Working standard solutions of zoxamide were obtained by appropriate dilutions of the stock solution with concentrations disregarding its GC sensitivity (Table 1) and were used as spiking solutions as well. Matrix matched standards were prepared at the same concentrations as that of the calibration solutions by adding the appropriate amounts of standards to the control matrix extracts.

2.2. Gas chromatography-ion-trap mass spectrometry

A Varian 3800 gas chromatograph with a Saturn 2000 (Varian, Walnut Creek, CA, USA) ion trap mass selective detector (ITMS) was used. The gas chromatograph was equipped with a Varian 7800 auto sampler and a split/splitless, with a temperature program control, injector Varian 1079, operated in large volume injection mode. A DB 5 MS, 30 m × 0.25 mm i.d. capillary column with a 0.25 μm film thickness (J&W Scientific, Folsom, CA, USA), was used. The glass liner contained

Table 1
Recoveries (% ±SD) of zoxamide in grapes must, wine, and spirits at different fortification levels (mg/kg)

Fortification level (mg/kg)	Recovery (% ±SD)			
	Grapes	Must	Wine	Spirits
0.05	112.1 ± 5.9	80.4 ± 3.2	89.0 ± 6.0	99.1 ± 2.5
0.10	92.6 ± 4.3	96.5 ± 4.7	91.0 ± 13.0	97.4 ± 6.3
0.50	87.0 ± 8.0	114.0 ± 3.0	86.0 ± 9.6	95.8 ± 4.6
2.00	106.2 ± 3.2	90.0 ± 3.0	98.6 ± 1.6	100.0 ± 13.0

a Carbofrit (Restek, Bellefonte, PA, USA) plug. The carrier gas (helium) flow rate operate in the constant flow mode at 1.0 mL/min. A Varian 8200 autosampler was used to perform 8 μL injections. The ion trap mass spectrometer was operated in electron ionization (EI) and chemical ionization (CI, acetonitrile) mode. Helium (purity 99.999%) was used as carrier gas.

2.2.1. Instrumental conditions

The injector temperature was programmed at 60 °C (hold 1 min), to 150° at 30 °C min^{-1} (hold 20 min). The injector operates in splitless mode with purge valve on at 2 min. The carrier gas operates at 1.0 mL/min in constant flow mode. The oven temperature ramped from 65 °C (hold 1 min), to 280 °C at 10 °C min^{-1}. Total time for GC analysis was 22.5 min. The mass spectrometer was calibrated weekly, following the autotune test of the software (Saturn GC/MS Workstation 5.41). Two ionization modes (CI and EI) were used. Air and water were checked daily as well as the pressure of CI solvent. The common parameters for both mode were multiplier voltage (1×10^5 gain) of 1400 V, multiplier offset +100. Trap, manifold and transfer line temperatures were at 170, 100 and 200 °C, respectively. Automatic gain control (AGC) was turned on. For CI the emission current was set to 30 μA, prescan ionization time was 100 μs and AGC target value was 2000 counts. In EI mode the same values were 80 μA, 1500 μs, respectively. Selective ion storage (SIS) in the range mode (*m*/*z* 170–260) option was used for EI, while SIS *m*/*z* 300 was used for CI experiments.

The MS/MS experiments were carried out with the following conditions, waveform resonant: parent ion *m*/*z* 299.

2.3. Sample collection and storage

Samples of grapes of the cultivar Cabernet, were harvested at ripening, and processed to wine used a winemaking scheme previously described [6]. The wine, the lees, and the cake obtained during the wine making process of blank grapes were submitted to the distillation process. Samples were stored at 4 °C and submitted to GC-ITMS analysis within 24 h.

2.4. Analytical procedure

2.4.1. Distillation products

Spirits were distilled using the Jaulmes apparatus (Leqeaux, Paris). The alcoholic content was measured using a hydrostatic balance Densimat (Gibertini Elettronica, Milan, Italy). The study was carried out fortifying the wine, the lees, and the cake with the pesticide according to the higher residue found in wine after field experiments (about 0.20 mg/kg). To produce brandy or grappa, lees and cake are usually submitted to a first distillation in a stripping column, which produce a spirit of about 30% of alcohol, followed by a second distillation in a rectifying column, to give a spirit of about 80% alcohol [7]. In our experiment we tried to reproduce the same scheme, by producing a distilled spirit at alcoholic content similar to that of the industrial process.

2.4.2. Culture media

2.4.2.1. Yeasts. Zoxamide was dissolved in ethanol (5 mL) and added to 1 L of YNBG broth, made up of 7 g/L yeast extract nitrogen base (YNB) and 180 g/L glucose (G) at pH 3.6. All media were sterilized by filtration through 0.22 μm membrane filters (Millipore, Milan, Italy).

2.4.2.2. Bacteria. Zoxamide was dissolved in ethanol (5 mL) and diluted to 1 L with Vermentino wine containing 12% ethanol, 0.45 g/L volatile acidity, 6.2 g/L total acidity, and 4.40 g/L malic acid. The wine was sterilized by filtration through 0.22 μm membrane filters (Millipore, Milan, Italy).

2.4.3. Inoculation and fermentation

2.4.3.1. Yeasts. The yeasts were *Saccharomyces cerevisiae* strain 1090 VER 1 and *Kloeckera apiculata* strain 3197 MON 2 from the collection of the Dipartimento di Scienze Ambientali Agrarie e Biotecnologie Agroalimentari, Universita' di Sassari, Italy. Precultures were prepared in YNBG broth made up of 7 g/L yeast extract nitrogen base (YNB) and 5 g/L glucose (G) at pH 3.6 and agitated in a rotary shaker at 120 rpm for 48 h. The cells were then washed twice and suspended in 0.15 M sodium chloride. The amounts of the suspensions used as inocula were chosen to ensure 5×10^6 cells/mL in each of the culture media. After inoculation, the culture media of each strain and each pesticide were divided into three 150 mL replications in 500 mL glass flasks. Two different controls were prepared, consisting respectively of 150 mL of each culture medium without inoculum (YNBG plus zoxamide) to check pesticide chemical degradation, and inoculated (YNBG broth without zoxamide) to check fermentation. Each experiment was carried out in triplicate. All flasks were rotary agitated in a thermo-statically controlled chamber at 20 °C for 11 days. Three samplings were carried out at 0, 4 and 11 days after inoculation. The following analyses were made: pH, yeast cells per milliliter (microscopic and cultural count), and CO_2 production (indirect weighing).

2.4.3.2. Bacteria. Two commercial lactic bacteria, *Leuconostoc oenos* and *Oenococcus oeni*, were used. Precultures were prepared in MRS (Oxoid, Hampshire, UK) broth for 5 days without agitation and inured to alcohol (increasing rate by 5, 7 and 10%). The amount of suspension used, as inocula were chosen to ensure 5×10^9 cells/mL in wine. For each strain, after inoculation, 150 mL of wine was apportioned in 500 mL flasks. All of the flasks were allowed to incubate in a thermostatically controlled chamber at 32 °C. To check the fermentative activity due to acid malic degradation and pesticide stability, two different controls were prepared consisting of wine with inoculum, and wine with pesticide, respectively. Sampling was carried out at 0, 10, 20 and 30 days after inoculum. Each experiment was carried out in triplicate.

2.4.4. Extraction procedure

One kilogram grape sample was chopped with an appropriate chopper (Malavasi, Bologna, Italy). A 5 g aliquot of homogenized grape samples was weighed in a 40 mL screw-capped flask plus 2 g of NaCl and extracted with 10 mL of hexane. The

tubes were then agitated in a rotary stirrer for 15 min. The phases were allowed to separate, and the organic layer was poured into another screw-capped tube containing 1 g of anhydrous sodium sulfate. The organic layer was injected for GC analysis. Wine making was carried out on 5 kg grape sample, 5 mL of must, wine, and spirits, were weighed in a 40 mL screw-capped flask and processed according the grape extraction procedure.

2.4.5. Recovery assay

Untreated grape, must, wine, and distilled spirit samples were spiked prior to extraction by the addition of the appropriate volume of stock standard solution to reach 0.05, 0.10, 0.50 and 2.00 mg/kg of zoxamide, and processed according to the above-described procedure (Section 2.4.2). The matrix effect was evaluated by comparing the analytical response of the pesticide dissolved in acetone/hexane and in control matrix extracts.

3. Results and discussion

3.1. GC ITMS determination

The GC-ITMS method was used for the determination of zoxamide in grapes, must, wine, and spirits. Using a carbofrit inserted into the glass liner and large volume injection (8 μL) enhanced the sensitivity of the method [8–10]. The trap was kept off for the first 5 min in order to avoid damages due to the high volume of solvent injected. Analyses were performed both in CI and EI mode. Zoxamide molecular weight is 336.65 amu, in CI mode with acetonitrile as solvent, gave two granddaughter ions (Fig. 1) of 300 (base peak), and 302 amu, accounting for the 100 and 61% of relative abundance, respectively. Since these two ions represent the cluster of a biclorine molecule, the loss of 36 amu from the parent compound is justified by a neutral loss of HCl (Table 2). The fragmentation pattern in EI mode is reported in Fig. 1 and Table 2. Zoxamide showed in the position 1 of the dichloro methyl benzene ring a side chain with three different sensible sites of fragmentation. MS/MS experiments elucidate fragments daughter origins in EI mode: zoxamide did not show the molecular ion since an immediate cleavage of a radical H and successively of a neutral HCl, produced the ions of 335 *m*/*z*, and 299. The granddaughter ions of 242 and 187 *m*/*z* belong from the ion of 299 *m*/*z* and from the molecular ion, respectively. The ion of 299 *m*/*z* loses CO and led to the formation of an ion species of 271 *m*/*z*, while the ion of 242 *m*/*z* lose CO and gave the ion at *m*/*z* 214. Calibration graph were determined comparing peak height versus concentrations. A good linearity was achieved in the range 0.05 and 3.00 mg/kg, with a correlation coefficient above 0.9996.

Table 2
Intra- and inter-day reproducibility of three different standard concentrations in EI and CI mode

Standard concentration (mg/kg)	Reproducibility CV			
	Intra-day		Inter-day	
	Retention time	Height	Retention time	Height
EI				
0.13	0.03	3.37	0.07	11.88
0.60	0.01	5.13	0.08	9.37
1.30	0.01	4.20	0.11	9.12
CI				
0.13	0.06	17.26	0.17	26.44
0.60	0.18	4.43	0.17	14.13
1.30	0.03	9.73	0.04	11.47

The extraction procedure allows recoveries (Table 1) ranging from 98 and 101% with a maximum coefficient of variation (CV) of 14%. The limit of detection according to Thier e Zeumer [11] was 0.01 mg/kg both in EI and in CI mode. The limit of quantification (S/N = 10) was 0.05 mg/kg in EI and 0.08 mg/kg in CI, respectively.

3.2. Alcoholic fermentation

Trials were carried at two concentration 0.25 and 1.50 mg/kg, corresponding to the maximum concentration found on grapes and wine, respectively. Table 3 reports the effect of zoxamide on the fermentation activity of *S. cerevisiae* and *K. apiculata*. From the data here reported can be assumed that zoxamide did not affect the fermentation process. Table 4 reports zoxamide residues (mg/kg ± SD) during alcoholic fermentation of *S. cerevisiae* and *K. apiculata* yeasts. Both yeasts produced a decrease on pesticide residues, higher at high concentrations of zoxamide. Moreover *K. apiculata* was more effective compared to *S. cerevisiae* in decreasing residues levels at higher levels of zoxamide 43 versus 35%.

Table 3
Effect of zoxamide on fermentation activity of *Saccharomyces cerevisiae* and *Kloeckera apiculata*

Pesticide added (mg/L)	0			4			10		
	Cell/mL	pH	CO_2	Cell/mL	pH	CO_2	Cell/mL	pH	CO_2
Saccharomyces cerevisiae days after inoculation									
Control	5.0×10^6	3.6	n.d.	6.0×10^7	3.3	5.5	7.0×10^7	3.7	7.5
0.25	5.0×10^6	3.6	n.d.	5.8×10^7	3.3	5.0	6.8×10^7	3.7	6.5
1.50	5.0×10^6	3.6	n.d.	6.2×10^7	3.3	5.5	7.5×10^7	3.6	6.7
Kloeckera apiculata days after inoculation									
Control	5.0×10^6	3.6	n.d.	5.8×10^7	3.5	3.5	6.6×10^7	3.6	6.5
0.25	5.0×10^6	3.6	n.d.	6.7×10^7	3.5	3.2	6.8×10^7	3.7	4.7
1.50	5.0×10^6	3.6	n.d.	6.4×10^7	3.4	3.0	6.5×10^7	3.7	4.0

Table 4
Zoxamide residues (mg/kg ± SD) during alcoholic fermentation of *Saccharomyces cerevisiae* (S) and *Kloeckera apiculata* (K) yeasts

Sample	Residues after inoculation			Percent of decrease (%)
	0 days	4 days	10 days	
Control (0.25 mg/kg)	0.24 ± 0.02		0.28 ± 0.04	0
S	0.26 ± 0.05	0.18 ± 0.01	0.18 ± 0.01	30
K	0.23 ± 0.01	0.20 ± 0.01	0.15 ± 0.00	22
Control (1.50 mg/kg)	1.31 ± 0.10		1.21 ± 0.09	
S	1.41 ± 0.09	1.26 ± 0.07	1.10 ± 0.06	35
K	1.44 ± 0.03	1.00 ± 0.03	0.81 ± 0.03	43

Table 5
Malic acid (g/L ± SD) during malolactic fermentation of *Leuconostoc oenos*, and *Oenococcus oeni* lactic bacteria

Pesticide added (mg/L)	0 days	10 days	20 days	30 days
Leuconostoc oenos, after inoculation				
Control	4.03 ± 1.03	5.47 ± 0.30	5.51 ± 0.22	5.15 ± 0.86
0.5	4.28 ± 0.92	5.11 ± 0.30	4.90 ± 0.01	5.22 ± 0.38
3	4.95 ± 0.08	5.23 ± 1.05	4.58 ± 0.17	4.98 ± 0.97
Oenococcus oeni, after inoculation				
Control	4.27 ± 0.29	5.04 ± 0.37	5.17 ± 0.15	5.08 ± 0.19
0.5	4.44 ± 0.10	5.13 ± 0.20	5.19 ± 0.22	5.35 ± 0.26
3	5.14 ± 0.14	5.37 ± 0.31	5.15 ± 0.21	4.80 ± 0.32

Table 6
Zoxamide residues (mg/kg ± SD) during malolactic fermentation of *Leuconostoc oenos*, and *Oenococcus oeni* lactic bacteria

Pesticide (mg/L)	Bacteria	Days after treatment			
		0	10	20	30
0.50	Control	0.51 ± 0.18	0.57 ± 0.09	0.41 ± 0.01	0.32 ± 0.11
	Leuconostoc oenos	0.49 ± 0.10	0.46 ± 0.04	0.34 ± 0.08	0.36 ± 0.05
	Oenococcus oeni	0.50 ± 0.012	0.31 ± 0.00	0.31 ± 0.03	0.32 ± 0.05
3.00	Control	2.91 ± 0.34	3.30 ± 0.28	3.06 ± 0.36	3.06 ± 0.46
	Leuconostoc oenos	2.80 ± 0.26	3.34 ± 0.36	2.98 ± 0.22	2.84 ± 0.12
	Oenococcus oeni	3.05 ± 0.26	3.37 ± 0.39	3.06 ± 0.25	2.68 ± 0.21

3.3. Malolactic fermentation

Table 5 reports the malic acid levels (g/kg ± SD) during malolactic fermentation of *L. oenos*, and *O. oeni* lactic bacteria. The different concentrations of fungicides did not affect the behavior of the bacteria. Table 6 shows pesticide residues behavior during malolactic fermentation. Considering the average values the trend showed a reduction of residues level during the experiment time, but this decrease was not statistically different.

3.4. Repeatability

Intra-day and inter-day repeatability of retention times and heights of six consecutive injection in six different days, sequences of 8 μL spiked blank and of a standard at three different concentrations 1.30, 0.60, and 0.13 mg/kg are shown as % relative standard deviation (CV; Table 2).

4. Conclusions

The reported method allows a simple and rapid determination of zoxamide in grapes, wine and spirits. Since the GC–MS chromatograms of untreated matrix extracts were all free from interfering peaks and indistinguishable from those obtained with pure solvents, no clean up was needed.

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