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Short communication

Influence of the presence of ethanol on *in vitro* bioavailability of fungicide residues

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ABSTRACT

The influence of ethanol on the *in vitro* bioavailability of nine fungicides in wines of varying degrees of alcohol is studied by simulating the digestive process by dialysis in semipermeable membranes. The dose of each fungicide corresponds to its added Maximum Residue Limits in the different matrices (water, ethanol, wines of 7, 11, 13 and 14.5% ethanol). A validated analytical methodology was used which includes extraction, partition according to the modified QuEChERS multiresidue method and liquid chromatography with triple quadrupole tandem mass spectrometry analysis. Interaction between the ethanol content and bioavailability was confirmed and increases were found in the dialyzed percentages with respect to the blank (the standard in water) for ametoctradin, mepanipyrim, cyazofamid, pyraclostrobin and metrafenone, while a decrease was observed for dimethomorph, boscalid and kresoxim-methyl. Fenhexamid showed no significant differences by alcohol content.

1. Introduction

Given the importance of pesticides in agriculture and public health activities, it is impossible to deny the toxic effects that these can produce in humans. The still important number of pesticides in use today makes it imperative that controls be carried out which identify and measure the residues of these compounds in food produce. Considering the importance of the constituents of the Mediterranean diet, and wine in particular, which apart from its physico–chemical characteristics, can lead to residues being transferred to the must, and from there to the finished product, and can even affect its organoleptic quality if precautions are not taken and the vinification is not correctly performed (Navarro et al., 1999; García et al., 2004; Fernández et al., 2005a, 2005b; Oliva et al., 2008; Oliva et al., 2011, 2014; Payá et al., 2013; Mulero et al., 2015). Clearly this is a source of great concern to the consumer of the final product and so studies have been made on the exposure and ingestion of foods in order to control food safety aspects properly (Badii and Valera, 2008). Among these controls, calculating the bioavailability of the pesticide residues present in foods is considered to be of great interest. Bioavailability is the amount of a substance which, after ingestion, is absorbed and enters the systemic circulation, from where it is distributed and can reach the target tissues, where it exerts its physiological effect (Parada and Aguilera, 2007). Two models are generally used to study oral bioavailability in drugs and xenobiotics: a) *In vivo,* using animals in experiments that are easily correlationable with human beings, but which pose problems in terms of cost, time and ethics; b) *In vitro*, using semi-permeable membranes and appropriate methods to simulate the digestion process. The *in vitro* models are acceptable at the initial stage of research as they a good indication of what occurs physiologically and are much more economic, simpler and faster (Bollinger et al., 2005; Payá et al., 2009a, 2013, 2009b). It has been shown that the ethanol content of a matrix has a notable influence on the stomach's absorption of certain cations and organic components, and there is evidence that the degree of alcohol and the content of the phenolic compounds can increase bioavailability in red wines (Monteiro et al., 2005; McRae et al., 2015; Shishan et al., 2015). Knowing the bioavailability of a compound is fundamental in order to know the effect it will have on the body and to determine the amount necessary to establish the minimum and maximum amounts between which it can have an effect. It is therefore essential to have a study of the bioavailability of a substance embedded in the medium in which it will enter the body and so be able to determine exactly how much penetrates the body. We believe that it is of interest to study and compare the bioavailability of nine fungicides widely used in vineyards in wines of different degrees of alcohol compared to that in pure water and pure ethanol.

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2. Materials and methods

2.1. Materials

2.1.1. Pesticides

Dimethomorph,(*E,Z*)-4-[3-(4-chlorophenyl)-3-(3,4-dimethoxyphenyl)acryloyl] morpholine (MRL = 3 mg kg^{-1}); kresoxim-methyl: methyl (*E*)-methoxyimino[2-(*o*-tolyloxymethyl)-phenyl]acetate (MRL = 1 mg kg^{-1}); mepanipyrim: N-(4-methyl-6-prop-1-ynylpyrimidin-2-yl)aniline (MRL = 2 mg kg^{-1}); metrafenone: 3′-bromo-2,3,4,6′-tetramethoxy-2′,6-dimethylbenzo-phenone (MRL = 5 mg kg^{-1}); cyazofamid: 4-chloro-2-cyano-N,N-dimethyl-5-p-tolylimidazole-1-sulfonamide (MRL = 0.5 mg kg^{-1}); boscalid: 2-chloro-N-(4′-chlorobiphenyl-2-yl)nicotinamide (MRL = 5 mg kg^{-1}); fenhexamid: 2′,3′-dichloro-4′-hydroxy-1- methylcyclohexane−carboxanilide (MRL = 5 mg kg^{-1}); pyraclostrobin: methyl N-{2-[1-(4-chlorophenyl)-1H-pyrazol-3-yloxy-methyl]phenyl}(N-methoxy) carbamate (MRL = 2 mg kg^{-1}); ametoctradin: 5-ethyl-6-octyl[1,2,4] triazolo[1,5-a]pyrimidin-7-amine (MRL = 6 mg kg^{-1}). All the products were obtained from Dr. Ehrenstorfer (Ausgburg, Germany), with a purity of 98%.

A stock solution was prepared of 1000 mgl^{-1} of each of the nine fungicides in acetonitrile. A 50 ml working solution was then prepared containing the necessary concentration of each fungicide sothat on fortification with 1 ml the samples undergoing dialysis would attain the MRL value for each fungicide.

2.1.2. Matrices

The fortified samples were water, ethanol 96% v/v and commercial red wines of varying alcohol degrees - 7, 11, 13 and 14.5%.

2.1.3. Reagents and solvents

Ethanol 96% v/v. Panreac (Barcelona, Spain). Sodium sulfate anhydro with purity of 99%, Panreac (Barcelona, Spain); sodium bicarbonate, with purity of 99.0%, Scharlau (Barcelona, Spain); pepsin from hog stomach mucous, Sigma Aldrich Chemie (St. Louis, Missouri); solution of 4 g of pepsin in 25 ml of HCl 0.10 M; hydrochloric acid, Scharlau (Barcelona, Spain); bile salts, Sigma Aldrich Chemie (St. Louis, Missouri); pancreatin from porcine pancreas, Sigma Aldrich Chemie (St. Louis, Missouri); solution of 0.4 g of pancreatin, 2.5 g of bile salts and 0.84 g of sodium bicarbonate in 100 ml of millipore water; dialysis membrane with 12.000 Da, Sigma (St. Louis, Missouri); sodium hydroxyde of 97% purity Scharlau (Barcelona, Spain); acetonitrile grade HPLC, Scharlau (Barcelona, Spain) and millipore water, Millipore Purification Pak (Billerica, Massachusetts); ammonium formate, of 95% purity, Fluka (Buchs, Suiza); anhydrous magnesium sulfate, of 97% purity, Fluka (Buchs, Suiza); sodium chloride, of 99.5% purity, Fluka (Buchs, Suiza); disodium citrate sesquihydrate, of 99% purity, Aldrich (Milwaukee, USA) and trisodium citrate dihydrate, of 99% purity, Sigma (St. Louis, USA); ethanol, of 96% with purity, Scharlau (Barcelona, Spain).

2.1.4. Apparatus

pH-meter, Crison (Barcelona, Spain); Shaking water bath at constant temperature, Julabo (Seelbach, Germany); HPLC Agilent Technologies model 1260 Infinity (HPLC-MS/MS QqQ) equipped with a Poroshell 120 EC-C18 (3 mm × 100 mm × 2.7 μm) column coupled to a triple quadrupole mass spectrometer 6410B equipped with an ESI (electrospray ionization) source in positive mode; all of these from Agilent Technologies (Palo Alto, California); centrifuge HERAEUS, Mod. Multifuge 3L-R (Hanau, Germany).

2.2. Methods

2.2.1. Extraction procedure

Extraction and partition was by a modified version of the QuEChERS multiresidue method (Martinez et al., 2015; Payá et al., 2007a), based on the extraction of the matrix with acetonitrile followed by the addition of the corresponding salts; the separated extract can be directly injected into the chromatograph by acidification with formic acid.

2.2.2. LC-MS/MS analysis

Chromatographic separation of nine fungicides was performed on an Agilent (Palo Alto, California, USA) 1260 Infinity Series system consisting of an autosampler, a binary solvent pump and column heater equipped with a Poroshell 120 EC-C_{18} (3.0 × 100 mm, 2.7 μm) from Agilent Technologies (Palo Alto, California, USA). The mobile phase consisting of 0.1% (v/v) formic acid in acetonitrile (solvent A) and 0.1% formic acid and 2 mM of ammonium formate in water (solvent B) was pumped at a flow rate of 0.6 ml min^{-1}. The gradient program was started with 20% component A (80% B) at injection time and increased linearly to 100% A in 10 min, then returned to the initial conditions in 2 min. The column oven temperature was maintained at 40 °C and the sample volume injected was 5 μl. A triple quadrupole mass spectrometer 6410B (Agilent Technologies, Palo Alto, California, USA) equipped with an ESI source was applied to quantify these fungicides. The nebulizer gas was nitrogen and the collision gas was argon. MS/MS detection was performed in positive ion mode, and the monitoring conditions were optimized for target compounds. The conditions were typically as follows: the capillary voltage was set as 3000 V while the source temperature and desolvation temperature were held at 120 °C and 350 °C, respectively. A 1 l min^{-1} cone gas flow and 9 l min^{-1} desolvation gas flow were used. Multi-reaction monitoring (MRM) was used for the detection of all compounds with a cycle time of 500 ms. All other ESI and MS parameters were optimized individually for each target compound.

2.2.3. In vitro bioavailability trial

The method used to study the bioavailability was an *in vitro* process that simulated digestive activity. Pepsin was added to the sample to simulate the first digestion and then a new digestion was performed with pancreatin and bile salts. Dialyzation was performed using a semipermeable membrane (Payá et al., 2013).

2.2.4. Statistic treatment

The data were treated statistically. First, a test for homogeneity of variances (Levene's test), indicative of the type of analysis to use (parametric or non-parametric), was applied. For all the parameters studied, parametric analysis of the variance (ANOVA of one factor and factorial ANOVA) was complemented with a practical interpretation, the Eta squared index (Eta^2), by applying the test of significant minor difference (SMD). The treatment was performed using the SPSS 15.0 software application for Windows.

3. Results and discussion

The dialyzation percentages of the fungicides were compared in commercial red wines of various degrees of alcohol against the same process in pure water and pure ethanol. Table 1 shows the mean values, the standard deviation and the significant differences in the 6 assays.

In wines with an alcohol content of over 11% the highest dialysis percentages were found for fenhexamid and cyazofamid (3.5−4.5%), followed by ametoctradin, boscalid and kresoxim-methyl (2.2−2.6%), while the lowest were for metrafenone, pyraclostrobin, and dimethomorph (1.5−1.92%).