Journal of Chromatography A 1644 (2021) 462124

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Supercritical fluid chromatography-mass spectrometric determination of chiral fungicides in viticulture-related samples

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ARTICLE INFO

Article history:
Received 16 November 2020
Revised 22 March 2021
Accepted 25 March 2021
Available online 30 March 2021

Keywords:
Fungicides
Enantiomeric fraction
Wine
Soil
Supercritical fluid chromatography

ABSTRACT

Supercritical fluid chromatography (SFC), combined with mass spectrometry (MS), was employed for the determination of five chiral fungicides, from two different chemical families (acylalanine and triazol) in wine and vineyard soils. The effect of different SFC parameters (stationary phase, chiral selector, mobile phase modifier and additive) in the resolution between enantiomers and in the efficiency of compounds ionization at the electrospray source (ESI) was thorougly described. Under final working conditions, chiral separations of selected fungicides were achieved using two different SFC-MS methods, with an analysis time of 10 min and resolution factors from 1.05 to 2.45 between enantiomers. In combination with solid-phase extraction and pressurized liquid extraction, they permitted the enantiomeric determination of target compounds in wine and vineyard soils with limits of quantification in the low ppb range (between 0.5 and 2.5 ng mL^{-1}, and from 1.3 to 6.5 ng g^{-1}, for wine and soil, respectively), and overall recoveries above 80%, calculated using solvent-based standards. For azolic fungicides (tebuconazole, myclobutanil and penconazole) soil dissipation and transfer from vines to wines were non-enantioselective processes. Data obtained for acylalanine compounds confirmed the application of metalaxyl (MET) to vines as racemate and as the R-enantiomer. The enantiomeric fractions (MET-S/(MET-S+MET-R)) of this fungicide in vineyard soils varied from 0.01 to 0.96; moreover, laboratory degradation experiments showed that the relative dissipation rates of MET enantiomers varied depending on the type of soil.

1. Introduction

Many pesticides employed in agriculture have a chiral structure; thus, the persistence of these compounds in crops, their degradation rates in agriculture soils and even their bioaccumulation in invertebrates and toxicities towards non-target organisms might be enantioselective processes [1].

Mildew and *botrytis* are major diseases impacting the productivity of vines. So, different families of fungicides have been designed and marketed to control these infections. Many of these compounds are chiral molecules. Among them, acylalanine and azoles are widely applied to vineyards for the prevention and the control of infections caused by *mildew* and *botrytis* fungi, respectively. Metalaxyl (MET), and in a lesser extent benalaxyl (BEN), are the most popular acylalanine fungicides. Although the fungicidal activity of the R-enantiomer is much higher than that of the S-form [2], currently, both compounds are still marketed as the racemate in addition to formulations enriched in the R-form. MET has been often reported in wine [3,4] and vineyard soils [5,6]; however, no data are available regarding the enantiomeric profiles of the compound in these matrices. The group of azolic fungicides gathers a large number of active molecules authorized for agriculture treatments. Among them myclobutanil (MYC), tebuconazole (TEB) and penconazole (PEN) are the most popular ones as regards viticulture applications. To the best of our knowledge, these compounds are marketed only as racemates. Their transfer factors from grapes to wine are lower than in case of MET [7,8]; however, they are more persistent in soils [9]. In this regard, the European Union (EU) has included TEB and PEN in the watch list of emerging environmental pollutants [10], for which data about their environmental distribution are required in order to estimate their risk quotients.

According to literature, the relative enantiomeric degradation rates of the above fungicides in crops and soil are matrix dependent. In this vein, the dissipation rates of MET isomers in soils varied largely depending on soil microbiota [11], with the stability of the R-form decreasing dramatically in alkaline soils [12]. Some re-

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https://doi.org/10.1016/j.chroma.2021.462124
0021-9673/

cent data point out to the fact that the low fungicidal activity MET-S might perturb the metabolism of mammals in a higher extent than the R-enantiomer [13]; thus, in addition to total concentration data the knowledge of enantiomeric fractions of this fungicide, as well as their time-course evolution, is a matter of concern. Wang and co-workers [14] have reported a faster dissipation for the S form of TEB than the R enantiomer in cabbage, whilst the opposite behavior was noticed in cucumber. Also, the enantiomeric degradation rates of MYC and TEB in soil have been related to their organic matter content, pH and other physico-chemical properties [15]. In summary, non-target effects and dissipation rates of chiral fungicides might change depending on the investigated organisms, the properties of each soil matrix, and the specific metabolism of each crop. To the best of our knowledge, little information is available related to the enantioselective accumulation of above fungicides in viticulture related samples. Zhang et al. [16] described a faster degradation of TEB-R in grapes than TEB-S; however, no data have been found regarding the enantiomeric fractions (EFs) of the compound in wine.

To date, most methods employed for the determination of chiral pesticides are based on liquid chromatography, either under normal or reversed-phase conditions [1,17]. Some limitations of chiral LC-based methods are either the use of isocratic conditions, often optimized for the separation of the enantiomers of single compound [15, 18, 19], or, in case of multianalyte procedures, the employment of slow gradients leading to analysis times above 60 minutes [20]. Since some years ago, pharmaceutical laboratories have upgraded their chiral separation methods from LC to supercritical fluid chromatography (SFC). Major advantages of the latter technique are reduction of the analysis time, due to the higher diffusivity and lower viscosity of supercritical CO_2, and save of large volumes of toxic organic solvents used in chiral LC separations when performed under normal phase conditions [21]. The combination between SFC and electrospray mass spectrometry (ESI-MS) has expanded the applicability of the technique to determine trace level compounds in complex extracts obtained from environmental and food samples, either using non-chiral or chiral columns [22–25].

Herein, we evaluate the performance of SFC-ESI-MS for the chiral separation of a selection of five fungicides, belonging to two different chemical families, often employed in the control of *mildew* and *botrytis* infections in vines. Their residues have been often reported not only in viticulture related samples, but in general in agriculture soils and other environments impacted by agriculture activities [26]. Moreover, azolic fungicides are regarded as an environmental threat and pinpointed as concerning pollutants for which environmental monitorization is recommended [10]. Thereafter the method is applied to the analysis of commercial wines and vineyard soils. The enantiomeric fraction (EF) data are employed to draw conclusions regarding the application form of acylalanine fungicides (as racemates or as preparations of the most active R enantiomer), and to investigate the existence of potential enantioselective dissipation processes during the wine making process and in the soil of different vineyards from the Northwest of Spain.

2. Material and methods

2.1. Standards, solvents and sorbents

Standards of MET, BEN, TEB, MYC and PEN, as racemates, were purchased from Sigma-Aldrich (Milwakee, WI, USA). Isotopically labelled compounds (MET-$^{13}C_6$, TEB-d_9 and MYC-d_4, as racemic solutions) were obtained from the same supplier. The R enantiomers of MET and BEN were also purchased from Sigma-Aldrich, whilst R and S forms of TEB were kindly supplied by Shangai Chiralway Biotech Co (Minhang District, Shangai, China). Individual solutions of the above compounds were prepared in methanol (MeOH). Racemic mixtures of fungicides, used to spike soil and wine samples processed through this study, were made in the same solvent. A mix of isotopically labelled compounds in methanol was added to soil and wine samples before extraction. These compounds were employed as surrogate standards (SSs) to compensate non-quantitative recoveries and/or changes on compounds ionization yield at the electrospray source (ESI). Calibration standards containing increasing concentrations of native compounds (0.5 - 100 ng mL^{-1}), and a fixed level of labelled compounds (100 ng mL^{-1}), were prepared in MeOH: ACN (50:50).

MeOH and ACN, both LC-MS grade purity, formic acid (FA, 98 %), NH_3 (12% solution in MeOH), and acetic acid were supplied by Merck (Darmstadt, Germany). Ultra-pure deionized water (18.2 MΩ cm^{-1}) was obtained from a Milli-Q Gradient A-10 system (Millipore, Billerica, MA, USA). Carbon dioxide (CO_2) was purchased from Nippon Gases (Madrid, Spain).

OASIS HLB 200 mg cartridges, employed for solid-phase extraction (SPE) of wine samples, were acquired from Waters (Milford, MA, USA). Diatomaceous earth, used during pressurized extraction of vineyard soils, was provided by VWR (West Chester, PEN, USA).

2.2. Samples and sample preparation

Wines were either purchased in local supermarkets, or obtained directly from regional wine production associations. Samples were maintained in the dark, at room temperature and SPE extractions were carried out immediately after opening wine bottles.

Soils were taken in seven vineyards, corresponding to three different *Designations of Origin* in Galicia (Spain). Samples used in this study corresponded to top soil (0-15 cm depth) collected in polyethylene bags, and transported immediately to laboratory. After removing coarse materials, samples were freeze-dried and sieved. The fraction below 2 mm was stored at -20 °C and employed for analysis. Samples used to measure the EFs of fungicides in vineyard soils were collected at the beginning of autumn and/or the end of winter; thus, fungicides were in contact with the soil since, at least, the end of the previous year summer. Soils employed in laboratory incubation studies were taken at the end of spring (middle June), within the year period that fungicides are sprayed on vineyards.

Sample preparation was performed using previously published procedures dealing with pressurized liquid extraction [9] and SPE [27] of vineyard soils and wines, respectively. In brief, soil samples (2 g) were spiked with the mixture of SSs (250 ng g^{-1}) and packed in 11 mL stainless steel cells containing 1 g of diatomaceous earth. The free volume above the sample, within the PLE cell, was filled with the same sorbent. Cells were pressurized at 1500 psi and compounds were extracted using a mixture of MeOH:ACN (70:30) at 80 °C, in a single cycle with a duration of 5 min [9]. This extract was concentrated and made up to 5 mL, using volumetric flasks, and stored at 4 °C. Wines (2 mL) were diluted with the same volume of ultrapure water, spiked with SSs (100 ng mL^{-1}) and passed through a SPE cartridge previously conditioned with ACN: MeOH (80:20) followed by a mixture of EtOH: H_2O (12:88), 2 mL each. After loading the diluted samples, the sorbent was rinsed with 3 mL of the EtOH: H_2O solution and dried using a stream of nitrogen. Compounds were recovered with a mixture of ACN: MeOH (80:20). The extract from the SPE cartridge (2 mL) was maintained at 4 °C until analysis. Both sample preparation procedures were previously combined with LC-MS as determination technique using a non-chiral column for compounds separation [9,27]. Before injection in the SFC-MS system, all extracts were passed through a 0.22 µm syringe filter.

2.3. Soil incubation experiments

In addition to data obtained for field samples (vineyard soils), the potential enantioselective degradation of fungicides in this matrix was re-evaluated in laboratory incubation assays. The physicochemical properties of the samples used in this series of experiments are given as supplementary information (Table S1). Fractions of 2 g from each soil (particle size below 2 mm) were transferred to 20 mL glass vials and spiked with a racemic mixture of the five compounds considered in this study (addition level 200 ng g^{-1}). One of the soils (sampling point 2, Table S1) was fortified only with BEN and PEN given that it contained relevant residues of the rest of compounds (from 50 ng g^{-1} for TEB to 250 ng g^{-1} for MYC). Water content in incubation vessels was adjusted to 20% of sample weight. After Vortex homogenization, vials were capped using Teflon lined septa. A needle was passed through the septum and a 0.45 µm pore size filter was connected on top of the needle. This setup permits to assess compounds dissipation under aerobic conditions, whilst it reduces water evaporation [28]. Vials were maintained at 20 °C, and retrieved in duplicate at pre-defined times (from 0 to 66 days). Control experiments were performed with sterilized fractions of each soil matrix, incubated for 66 days. Soil sterilization was performed heating the sieved samples to 170 °C for 90 min. Extraction of soil samples was carried as defined in section 2.2 after addition of SSs.

2.4. SFC-ESI-QTOF-MS determination conditions

Separation of chiral compounds was carried out using an Agilent 1260 infinity II SFC system (Wilmington, DE, USA) connected to a quadrupole time-of-flight (QTOF) instrument (Agilent model 6550) furnished with dual spray ion funnel ESI source. The mobile phase from the SFC system was mixed with the make-up solution and divided in two streams. One reaches the ESI source through a 1 m x 0.050 mm i.d. silica capillary. The second stream is connected to the back-flush pressure regulator (BPR), which is responsible to maintain the CO_2 under supercritical conditions.

The TOF-MS instrument operated in the 2 GHz mode, offering a typical spectral resolution of 16000 (calculated as FWHM at m/z 322.0481). The ESI source was set in positive mode, and the *m/z* axis was continuously recalibrated using reference ions at *m/z* 121.0509 and 922.0098. Nitrogen was employed as nebulizing (35 PSI) and drying gas (15 L min^{-1}, 200 °C) in the ionization source. The ESI needle and the fragmentor voltages were set at 3500 V and 380 V, respectively. During optimization of SFC conditions, the instrument was run in the MS mode, using the peak areas for the $[M+H]^+$ ion of each compound as response variable. Analysis of soil and wine samples was carried out in the product ion scan acquisition mode. In both cases, quantification ions were extracted using a mass window of 20 ppm centred either in their $[M+H]^+$ ion, or in the most intense product ion of each compound (Table 1).

The polysaccharide-based chiral columns evaluated for compounds separation were obtained from Phenomenex (Torrance, CA, USA). Column dimensions were 150 mm (length) x 3 mm (i.d.), 3 µm particle size. The tested phases were amylose and cellulose with phenyl carbamate bonded to methyl and/or chlorine substituents as chiral selectors. Through this manuscript, columns are termed as amylose-1 (3,5-dimethyl phenyl carbamate), amylose-3 (3-methyl-5-chloro phenyl carbamate) and cellulose-5 (3,5-dichlorophenyl carbamate). In the former case, the stationary phase is coated on silica particles, whilst amylose-3 and cellulose-5 phases are immobilized on silica. The assayed mobile phases consisted of CO_2 (phase A) combined with MeOH, or ACN (phase B) as modifiers, containing different additives, such as FA (0.1%), ammonium acetate (NH_4Ac, 5 mM) or NH_3 (0.1%). In all the cases, the flow of mobile phase was 1.5 mL min^{-1} and columns were maintained at 40 °C. As make-up solution, a mixture of MeOH:FA (99.5: 0.5) was used to enhance compounds ionization in the ESI source [29]. Under final conditions, two different chromatographic methods were employed. The enantiomers of MET, BEN and TEB were separated using the amylose-1 column. The mobile phases consisted of CO_2 (A) and MeOH, 5mM in NH_4Ac, (B) combined as follows: 0-1 min (2% B), 4-6 min (30% B), 6.05-10 min (2% B). The identity of the enantiomers of these fungicides was confirmed by injection of R-forms of MET and BEN, as well as R and S isomers of TEB. Chiral separations of MYC and PEN were performed with the cellulose- 5 column using ACN 5 mM in NH_4Ac as organic modifier. The mobile phase gradient was: 0-1 min (10% B), 4-6.5 min (30% B), 6.51-10 min (10% B). The identities of the enantiomers for these two fungicides were not elucidated; thus, they are simply referred as isomers 1 and 2 attending to their elution order.

2.5. Evaluation of enantiomeric fractions, matrix effects and accuracy

EFs of fungicides in the extracts from wine and soil samples were calculated as the ratio between the concentration corresponding to the earlier eluting isomer and the sum of concentrations for both enantiomers [30].

Matrix effects (MEs) were evaluated with the ratio between the slope of calibration curves for matrix-based standards (prepared with spiked extracts from wine or soil samples) and solvent-based standards. Normalized ratios around 100% correspond to similar ionization efficiencies in both cases. Values below and above 100% point out to signal suppression and enhancement, respectively.

The accuracy of the analytical procedure was estimated using spiked samples of red and white wines, and vineyard soil. Spiked and non-spiked fractions of the above samples were extracted in triplicate. Concentrations of each enantiomer in the obtained extracts were calculated using solvent-based standards. Accuracy was estimated as the ratio between the difference of concentrations measured for spiked (samples were fortified before extraction) and non-spiked fractions of the investigated matrix divided by the added value and multiplied by 100.

3. Results and discussion

3.1. Optimization of SFC parameters

Enantiomeric separations of selected compounds were investigated combining the chiral columns described in section 2.4 with MeOH or ACN as modifiers of supercritical CO_2. In this set of preliminary experiments, the percentage of modifier in the mobile phase was varied as follows: 2% (0-1 min), 30 % (4-7 min), 2% (7.1-10 min). The mobile phase flow rate was 1.5 mL min^{-1} and the temperature of the columns set at 40 °C. As a general trend, ACN showed a lower solvation efficiency than MeOH, leading to longer retention times than those observed with the latter modifier. In some cases, the separation efficiency of the column was also lower for ACN than for MeOH, as a consequence of wider peaks noticed for the former solvent. As regards separation of enantiomers, resolution factors (Rs) were column and modifier dependent.

The amylose-3 column provided Rs above 1.5 only for the enantiomers of BEN (obtained using MeOH as modifier), data not shown. Table S2 summarized Rs and baseline peak width values obtained using amylose-1 and cellulose-5 columns in combination with MeOH and ACN as modifiers. The latter column separated the enantiomers of BEN and PEN with any of both organic modifiers; moreover, partial separation of MYC forms ($R_S > 1$) was observed with ACN. On the other hand, this column did not resolve the enantiomers of MET and TEB. The separation efficiency and the

Table 1
Retention times, quantification ions, linearity (R^2 values) and instrument limits of quantification (LOQs) of the SFC-QTOF-MS system.

Compound	Column	Retention time (min)	Rs	Quantification transition (Collision energy, Ev)	Other product ions	Linearity (R^2, 1-100 ng mL^{-1})	LOQ (ng mL^{-1})	Slope ratio ($1^{st}/2^{nd}$ enantiomer)
[a]MET-S	Amylose-1	2.66	1.05	280.1543 (10) > 220.1332	192.1383; 160.1121;	0.9989	0.5	1.00
[a]MET-R (M)		2.79				0.9984	0.5	
[a]BEN-S		3.02	2.45	326.1751 (10) > 148.1121	208.1332; 91.0642	0.9991	1	1.01
[a]BEN-R (M)		3.32				0.9978	1	
[c]TEB-S		4.19	1.61	308.1524 (20) > 70.0399	125.0153	0.9995	0.5	0.99
[c]TEB-R		4.38				0.9983	0.5	
[b]MYC-1	Cellulose-5	4.63	1.25	289.1215 (20) > 70.0399	125.0153	0.9990	0.5	1.01
[b]MYC-2		4.8				0.9956	0.5	
[b]PEN-1		5.55	1.55	284.0714 (20) > 70.0399	158.9763	0.9977	2.5	0.94
[b]PEN-2		6.25				0.9962	2.5	
[a]Met $^{13}C_6$	Amylose-1	2.68; 2.81	1.04	286.175 (10) > 226.1531	198.1583; 166.1319			
[b]MYC-d_4	Cellulose-5	4.49; 4.68	1.26	293.1466 (20) > 70.0399	129.0397			
[c]TEB-d_9	Amylose-1	4.23; 4.40	1.59	317.2089 (20) > 70.0399	125.0153			

[a] Denote the surrogate standard associated to each compound.
[b] Denote the surrogate standard associated to each compound.
[c] Denote the surrogate standard associated to each compound.

enantiomeric selectivity of the amylose-1 column was heavily affected by the organic modifier. Using ACN, partial resolution (Rs values from 0.76 for MYC to 1.0 for PEN) was observed between the pairs of enantiomers of the 5 fungicides. However, their peak widths were 2-3 times larger than those noticed using MeOH. The latter modifier led to partial separation of the enantiomers of MET (Rs values around 1), the forms of BEN and TEB were baseline resolved, and no separation was noticed for PEN and MYC enantiomers.

The effect of different additives (NH_3 0.1%, FA 0.1% and NH_4Ac 5mM) in the performance of SFC separations was assessed using CO_2:MeOH and CO_2:ACN as mobile phases combined with amylose-1 and cellulose-5 columns, respectively. Triazolic fungicides are slightly basic compounds, so depending on the mobile phase pH, secondary interactions with the chiral stationary phase and/or with the silica particles might affect their SFC retention and separation [31]. The above additives did not modify the performance of SFC separations (efficiency, selectivity or resolution between enantiomers); however, they introduced significant effects in the efficiency of compounds ionization. Relative responses (normalized to those obtained without any mobile phase additive) varied depending on the compound and the SFC column (Fig. 1). For example, NH_3 (0.1%) combined with MeOH exerted a minor effect in the relative response found for MYC with the amylose-1 column, Fig. 1A; however, the responses for the enantiomers of this fungicide increased by a factor of 5 when the same additive was combined with ACN (Fig. 1B). The adopted compromise decision was to employ NH_4Ac (5 mM) as additive in combination with MeOH and ACN. This additive improved significantly the responses observed for the enantiomers of MET, BEN and MYC. On the other hand, the peak areas of TEB and PEN suffered a reduction in comparison to those attained without additive in the mobile phase. NH_4Ac also prevented differences in the responses for enantiomers of the same compound reaching the ESI source in a different environment, as regards the mobile phase pH. As example, the relative intensities of the chromatographic peaks for the enantiomers of BEN in the amylose-1 column, differed significantly when acid or basic additives are included in the mobile phase, (Fig. S1).

Another parameter considered during optimization of SFC conditions was the BPR pressure. Between 90 and 140 bar, retention times decreased slightly with increasing the pressure due to a higher polarity of the mobile phase. The effect of this parameter in the resolution of enantiomers was negligible and, as a general trend, responses (peak areas) increased significantly with BPR pressure, see Fig. S2. Thus, a working value of 140 bar was selected for this parameter.

Taking into account the above data, after slight modifications of the mobile phase gradient, two different chromatographic methods were proposed. Chiral determinations of MET, BEN and TEB were carried out in the amylose-1 column, using MeOH (5 mM in NH_4Ac) as modifier in the mobile phase. The percentage of modifier was programmed as follows: 2% (0-1 min), 30 % (4-6 min), 2% (6.05-10 min). For these three compounds, the earlier eluting isomer was the S-form. MYC and PEN were determined using the cellulose-5 column, with ACN (5 mM in NH_4Ac) as modifier. The content of modifier was varied as follows: 10% (0-1 min), 30 % (4-6.5 min), 2% (6.51-10 min). The elution order of the enantiomers of these compounds was not established. The cellulose-5 column permitted also the separation of BEN enantiomers, with a different selectivity to that reported for the amylose-1 column. That is, BEN-R eluted first than the S-form of the fungicide in the cellulose column. Under above conditions, maintaining chiral columns at 40 °C, the total pressure in the chromatographic system varied with the chromatographic gradient within the ranges of 210-250 bar (ACN), 200-250 bar (MeOH); thus, pressure remained 100 bar below the upper limit (350 bar) established for the employed chiral columns.

The effect of the make-up flow rate (MeOH: FA, 99.5: 0.5) in the responses of fungicides was evaluated in the range of values from 0.1 to 0.7 mL min^{-1}. Under chromatographic conditions employed with the amylose-1 column, the normalized responses of MET enantiomers and that of BEN-S increased significantly between 0.1 and 0.3 mL min^{-1} of make-up; thus, their ionization efficiencies improved with the flow rate of MeOH: FA (99.5: 0.5) reaching the ESI source (Fig. S3A). In case of BEN-R and TEB enantiomers, which elute from the column with a higher percentage of MeOH in the mobile phase, the effect of make-up flow was negligible. A working value of 0.3 mL min^{-1} was used in combination with this column. Under conditions employed in the cellulose-5 column, responses of all compounds decreased with the make-up flowrate, with the most dramatic effect observed for the enantiomers of PEN (Fig. S3B). Thus, a value of 0.1 mL min^{-1} was used in combination with this column. It is worth noting that, normalized responses of BEN enantiomers showed a different dependence with make-up flow rate as function of the modifier employed in the mobile phase (Fig. S3A and S3B). Thus, the composition of the

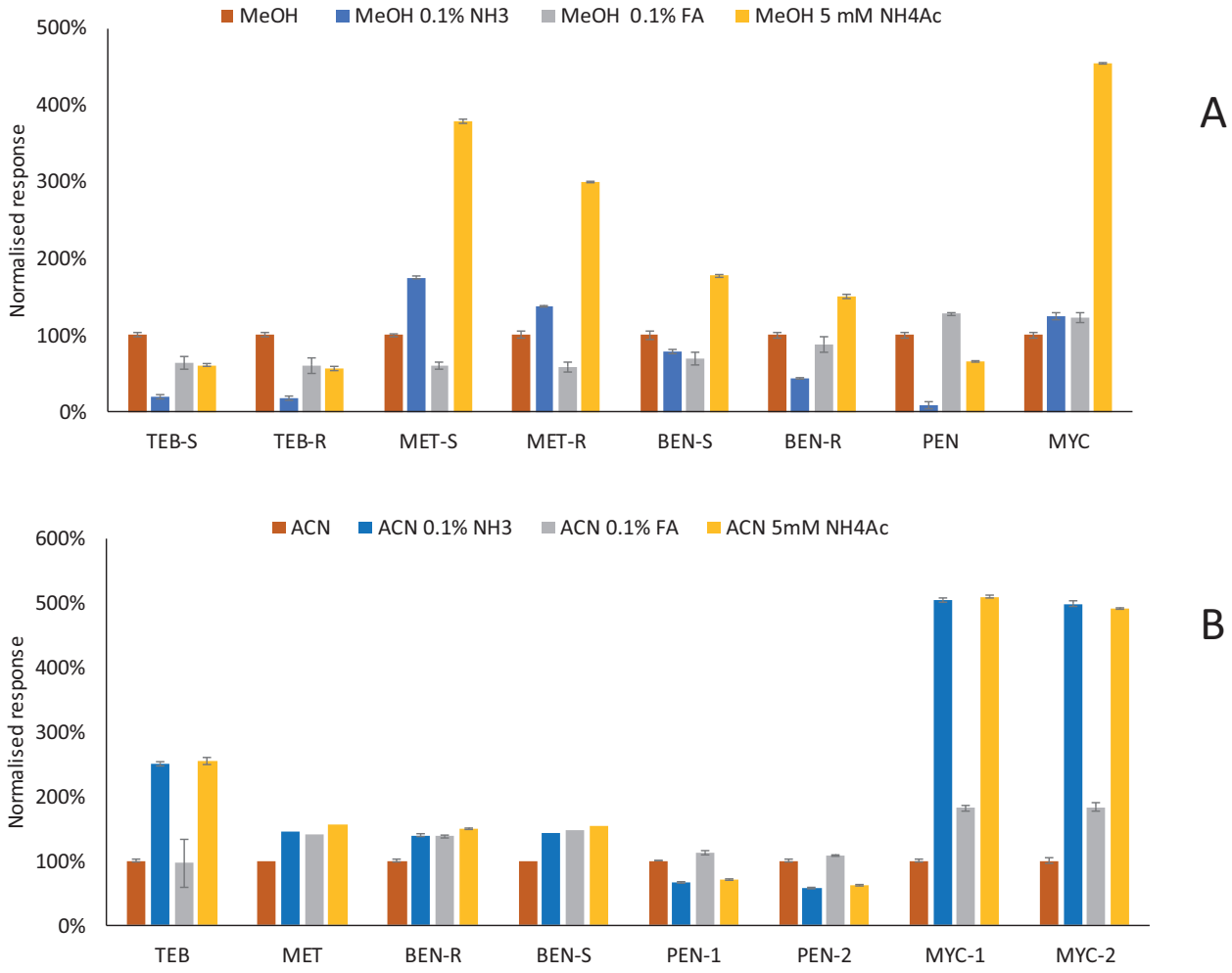

Fig. 1. Normalized responses as function of the mobile phase additive. A, amylose-1 column using methanol as modifier. B, cellulose-5 with acetonitrile as modifier, n=5 replicates.

CO_2: organic solvent reaching the ESI source plays a major effect in the efficiency of compounds ionization.

3.2. Characterization of the SFC-ESI-QTOF procedure

Table 1 compiles relevant data related to the performance of SFC-ESI-QTOF-MS methods considering the MS/MS detection mode. Linearity was investigated by injection of racemic mixtures of the above compounds prepared in MeOH. Within the range of concentrations from 1 to 200 ng mL^{-1} (values referred to the sum of enantiomers), linear responses were obtained for all the species with determination coefficients above 0.99. Limits of quantification, defined as the lowest concentration providing a signal to noise (S/N) of 10 for the quantification product ion varied from 0.5 ng mL^{-1} (enantiomers of MET, TEB and MYC) to 2.5 ng mL^{-1} (PEN enantiomers). These values are only slightly higher than those obtained in a previous study reporting the determination of same compounds by UPLC-QqQ-MS, using a non-chiral column (LOQs from 0.1 to 0.4 ng mL^{-1}) [27].

3.3. Matrix effects and accuracy assessment

The extraction yield of the sample preparation methods employed in the current study for wine and soil were characterized in previous publications of our group [9,27]. Thus, validation of the methodology described in this research was limited to the study of MEs, and the evaluation of the accuracy with spiked samples. Both variables are affected not only by sample preparation conditions, but also by the composition of the mobile phase in the ESI source, which differs between SFC and reversed-phase LC methods. The assessment of MEs demonstrated suppression of the ionization efficiency of certain compounds (Fig. 2). Particularly, the enantiomers of TEB and BEN showed a moderate signal attenuation for soil extracts and, in a lesser extent, during analysis of red wine. More significant than the magnitude of signal attenuation is the lack of differences between MEs observed for the enantiomers of the same species. This fact, reduces the risk of reporting false variations in their EFs when processing real samples.

The recoveries of the procedure, estimated using solvent-based standards, are given in Table 2. The spiked levels employed in this study were 20 and 50 ng mL^{-1} (case of wine) and 50 and 100 ng g^{-1} (soil). These values remain in the range of concentrations reported in commercial wines and vineyard soils [6, 9, 27]. Recoveries varied in the range from 80% to 117% with RSDs between 2 and 15%. The overall LOQs of the procedure are also compiled in Table 2. Reported values were estimated from instrumental LOQs (Table 1), considering sample amount and final extract volume for each type of sample, as well as signal attenuation effects observed for some compounds (Fig. 2). In the case of wines, the procedural LOQs are very similar to instrumental values. For soils, LOQs varied in the range from 1.3 ng g^{-1} to 6.3 ng g^{-1}.

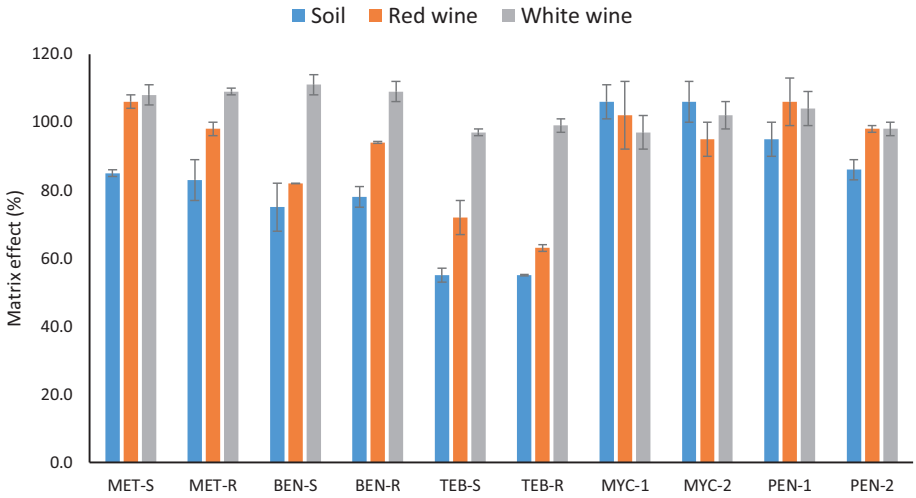

Fig. 2. Ratios between slopes of calibration curves for solvent and matrix-matched standards prepared using a pool of extracts from soil and wine samples.

Table 2
Overall recoveries, with standard deviations, for soil and wine samples spiked with racemic mixtures of compounds at two different concentration levels, n=3 replicates

Compound	Sample type						LOQs	
	Soil		Red wine		White wine		Soil	Wine
	50 ng g^{-1}	100 ng g^{-1}	20 ng mL^{-1}	50 ng mL^{-1}	20 ng mL^{-1}	50 ng mL^{-1}	(ng g^{-1})	(ng mL^{-1})
MET-S	98 (7)	108 (4)	103 (10)	91 (3)	117 (4)	107 (12)	1.5	0.5
MET-R	97 (8)	103 (7)	105 (12)	84 (3)	117 (5)	105 (12)	1.5	0.5
BEN-S	99 (8)	112 (5)	102 (15)	85 (3)	104 (5)	87 (12)	3.3	1.4
BEN-R	84 (7)	105(5)	106 (15)	80 (2)	110 (11)	97 (10)	3.3	1.4
TEB-S	93 (7)	107 (5)	104 (14)	89 (6)	111 (5)	107 (11)	2.3	0.7
TEB-R	98 (9)	112 (5)	107 (14)	94 (4)	110 (9)	105 (11)	2.3	0.7
MYC-1	105 (10)	99 (11)	99 (12)	91 (4)	114 (7)	104 (6)	1.3	0.5
MYC-2	100 (14)	110 (11)	97 (13)	91 (4)	108 (5)	108 (8)	1.3	0.5
PEN-1	108 (6)	92 (8)	95 (2)	88 (2)	96 (6)	110 (9)	6.3	2.5
PEN-2	94 (7)	104 (8)	97 (9)	88 (3)	106 (11)	106 (9)	6.3	2.5

Table 3
Enantiomeric fractions (EFs), with their standard deviations (SD), and average total concentrations of fungicides in commercial wines, n=3 replicates. R, red wine. W, white wine.

Sample code	MET			TEB			MYC		
	EF	SD	Conc. (ng mL^{-1})	EF	SD	Conc. (ng mL^{-1})	EF	SD	Conc. (ng mL^{-1})
R1	0.43	0.02	43	0.54	0.04	2	0.43	0.01	10
R2	0.05	0.09	8	0.52	0.01	37	0.42	0.02	4
R3	0.57	0.01	412	0.54	0.02	76	0.47	0.01	106
R4	0.56	0.01	344				0.44	0.01	18
R5	0.56	0.01	27						
R6	0.56	0.01	57						
W1	0.42	0.01	11						
W2	0.43	0.03	36						
W3	0.44	0.02	26						
W4	0.44	0.02	6						
W5	0.41	0.02	45	0.53	0.02	3			
W6	0.11	0.00	36	0.51	0.018	14			
W7	0.34	0.03	31	0.50	0.013	8			
W8	0.43	0.04	4	0.56	0.062	2			
W9	0.37	0.02	40	0.54	0.077	3			
W10	0.52	0.01	15						
W11	0.42	0.01	12	0.52	0.018	5			

Empty cells correspond to non-detected compounds.

3.4. Distribution of fungicides in wine and soil samples

Table 3 shows the total concentrations and the EFs of fungicides in a selection of 17 wines produced in Galicia (Northwest Spain). BEN and PEN remained below method LOQ in all samples, so these compounds are not included in the table. The detection frequency for the rest of fungicides increased in the following order: MYC < TEB < MET, with residues of the latter species found in all samples. Compared to the European Regulation for vinification grapes, the highest concentration of MET found in wine (412 ng mL^{-1}, equivalent to 412 ng g^{-1}, since the density of wine is around 0.994 g mL^{-1}) was close to 50% of its maximum residue

level authorized in vinification grapes (1000 ng g^{-1}) [32]. Globally, the EFs of TEB and MYC were equal to 0.5. This fact confirms that both compounds are commercialized as racemates and also, the absence of enantioselective dissipation processes either at vines, or during microbiological processes involved in must fermentation. In case of MET, the range of EFs varied from 0.05 to 0.57. EFs below 0.1, as that observed for wine code R2, likely correspond to grapes fumigated with the R-form of MET (commercialized under the name of MET-M). On the other hand, EFs slightly, although significantly, above 0.5 were measured in 4 red wines. Assuming that they were obtained from grapes treated only with the racemate of this fungicide, it seems that MET-S (the inactive fungicide isomer) is slightly enriched versus the R-form at vines and/or during wine elaboration. Obviously, confirming this assumption requires to the analysis of a relevant number of wines elaborated from grapes treated with the racemate of MET, with vinification developed under controlled conditions to avoid mixing in the same fermentation tank grapes, which received different treatments. Finally, in most white wines, EFs below 0.5 (0.37 to 0.43) were observed (Table 3). In this case, without information of vineyard treatments, it cannot be concluded a preferential accumulation of the R-form in this wine. The reason is that vines might have been fumigated with formulations including the racemate and also with other preparations containing just MET-M.

Average concentrations and EFs data for soil samples are summarized in Table 4. Samples were obtained from 7 vineyards from 3 *Designations of Origin* in Galicia (Northwest Spain). In this case, all fungicides were noticed in, at least, one of the investigated samples. Compounds dissipation was noticed in those points where pairs of samples were taken in autumn and at the end of winter (vineyards 1 to 4). Regarding EFs, those meassured for BEN, TEB and MYC were equal to 0.50; thus, no enantioselective degradation processes were identified. In case of PEN, EF values measured in October and March were equivalent in vineyards codes 1 and 2, although in vineyard code 1 a value below 0.5 was found in both sampling campaigns (Table 4). Finally, the EFs of MET, and their variation between samples obtained at different dates from same vineyard, differ as function of the sampling point. At vineyard code 3, MET-R was the predominant form in October without observing compound enantiomerization in March. EFs obtained for MET at vineyards 4 and 5 show a prevalence of MET-S. Since fungicidal preparations containing only MET-S are not commercially available, EFs above 0.5 are possible assuming a faster degradation of the R-form than that of S-isomer in these vineyards. On the other hand, at vineyards 1,2 and 7, the R-enantiomer was noticed at higher concentration than the S-form. In the particular case of vineyard 1, faster dissipation of MET-S compared to the R-form can be concluded from EFs measured in October and March (0.37 ± 0.01 and 0.28 ± 0.01, respectively). The SFC chromatograms for the most intense product ion of MET in soil samples showing different EFs are shown in Fig. 3.

3.5. Assessment of EFs under laboratory conditions

The potential existence of enantioselective degradation processes at vineyard codes 2, 3 and 4 (Table 4) was further assessed under laboratory conditions. Soil from these points were taken in June, in order to mimic microbiological conditions existing during the application period of these compounds, spiked with selected compounds and incubated under conditions reported in section 2.3. Table 5 summarized the total residual concentration of each fungicide at the end of the experiment, in non-sterilized and sterilized soils, normalized to that measured at day zero. TEB, MYC and PEN were hardly degraded during the experiment, whilst the

Table 4
Enantiomeric fractions (EFs), with their standard deviations (SD), and average total concentrations of fungicides in vineyard soils, n=3 replicates.

Vineyard code	Sampling date	MET			BEN			TEB			MYC			PEN		
		EF	SD	Conc. (ng g^{-1})	EF	SD	Conc. (ng g^{-1})	EF	SD	Conc. (ng g^{-1})	EF	SD	Conc. (ng g^{-1})	EF	SD	Conc. (ng g^{-1})
1	OCTOBER 2018	0.37	0.01	374										0.39	0.03	76
	MARCH 2019	0.28	0.01	96										0.40	0.03	27
2	OCTOBER 2018	0.30	0.05	14	0.52	0.01	14				0.50	0.01	1746	0.48	0.01	108
	MARCH 2019	0.35	0.04	7	0.50	0.01	7				0.51	0.01	586	0.50	0.03	50
3	OCTOBER 2018	0.01	0.01	408												
	MARCH 2019	0.03	0.01	71												
4	OCTOBER 2018	0.96	0.01	121												
	MARCH 2019	0.65	0.04	75												
5	MARCH 2019	0.62	0.03	13												
6	MARCH 2019	0.49	0.07	7			12	0.53	0.03	54	0.56	0.01	89			
7	MARCH 2019	0.27	0.03	29				0.50	0.02	164	0.44	0.02	146			

Empty cells correspond to non-detected compounds.

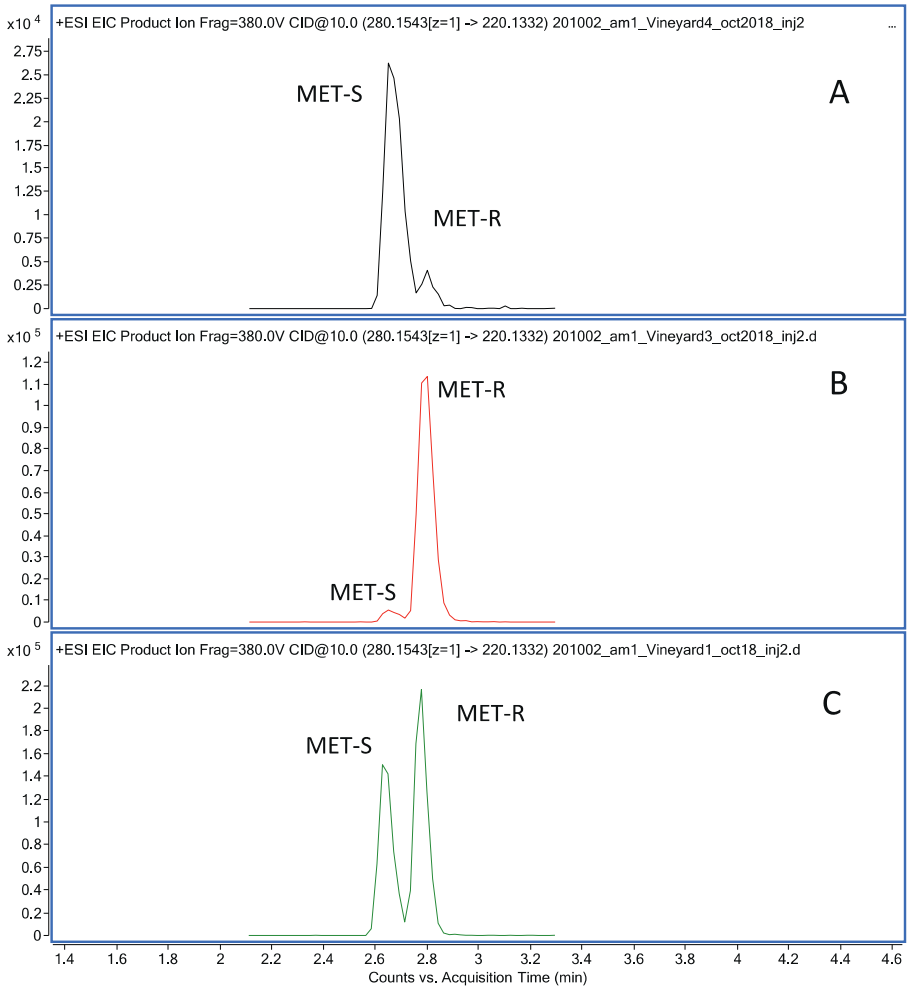

Fig. 3. Chromatographic profiles for the enantiomers of MET in soil samples obtained from different vineyards at the same date (October 2018). A, vineyard code 4. B, vineyard code 2. Vineyard code 1.

Table 5
Percentage of each fungicide remaining in soil after 66 days of incubation (n=2 replicates).

Vineyard soil code	MET		BEN		TEB		PEN		MYC	
	Aerobic	Sterilized	Aerobic	Sterilized	Aerobic	Sterilized	Aerobic	Sterilized	Aerobic	Sterilized
2	54%	108%	54%	92%	100%	99%	98%	100%	96%	105%
3	53%	89%	54%	92%	89%	84%	90%	86%	91%	97%
4	7%	90%	16%	92%	74%	92%	90%	113%	82%	89%

dissipated percentages of MET and BEN varied depending on the vineyard soil. In both cases, the lowest residue level was found in the same soil.

The average EFs at days 0 and 66 (n= 2 replicates) are given in Table S3. As expected, in case of compounds not removed during the experiment (TEB, MYC and PEN), EFs measured at days 0 and 66 were equivalent. For BEN, the EFs slightly decreased at day 66 compared to those calculate at zero time in soils from vineyards codes 2 and 3, but not in soil from vineyard code 4. The plot showing evolution of EF values for BEN and total compound concentration in the three soils involved in the study is provided as supplementary information (Fig. S4). Finally, the change in the EFs of MET depended on the soil matrix. Fig. 4 summarizes the time-course evolution of MET and the EFs of the compound during the incubation experiment. Samples from vineyards 3 and 4 were spiked with the racemate at 200 ng g^{-1} at day 0, whereas the initial concentration in the sample from vineyard code 2 corresponds to the native residue of MET existing in this soil. The kinetics of MET removal was sample dependent, with a much faster dissipation in soil number 4 (Fig. 4A), which matches the trend observed for BEN in same sample (Fig. S4). The evolution of the EFs of MET were also different between samples from vineyards codes 2 and 3, with a faster dissipation of MET-S (EFs decreased steady with incubation time), to that observed in vineyard soil code-4 (Fig. 4B). In the latter case, MET-R was degraded completeley after 14 days of incubation, leading to EF values close to 1. Thus, in agreement with data obtained under field conditions (Table 4), MET-R was less stable than MET-S in soil from vineyard code 4. Faster degradation of MET-R versus the S-form has been related to basic soils; however, the pH of soil obtained from vineyard code 4, and employed in the incubuation experiment, was slightly acidic, and intermediate between those corresponding to the other two samples involved in the same study (Table S1).

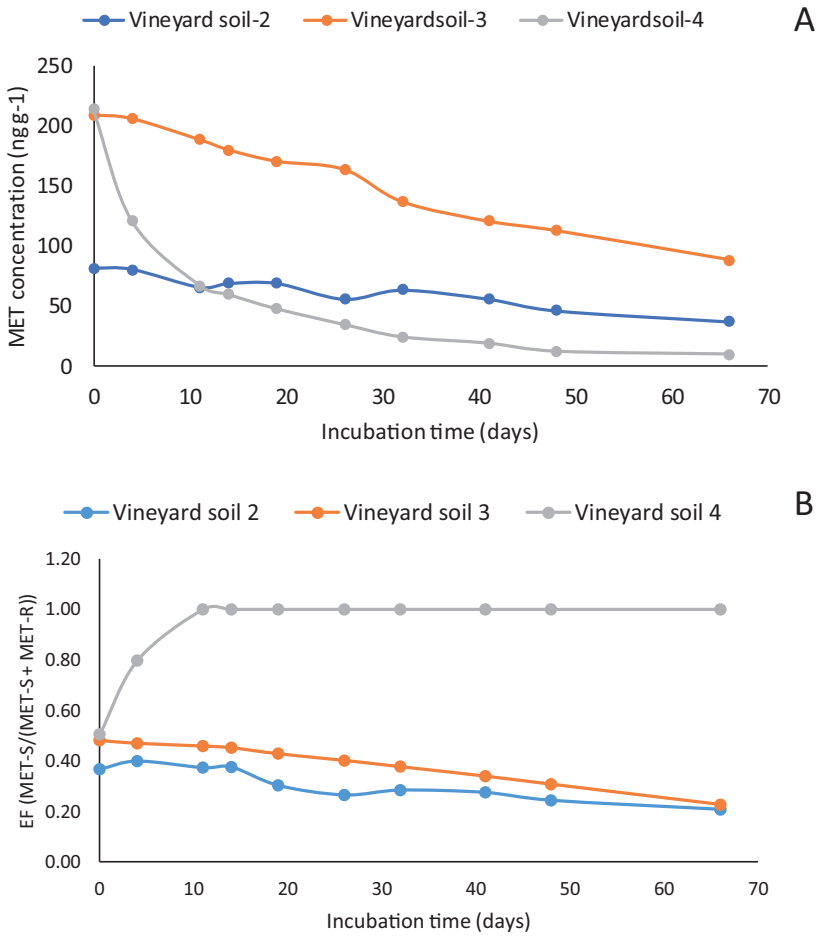

Fig. 4. Time-course evolution of MET in soils from 3 different vineyards in laboratory dissipation studies, average values of duplicate assays. A, total concentration. B, EF data (MET-S/(MET-S + MET-R). Soil from vineyards 3 and 4 were spiked with the racemic standard of MET (200 ng g^{-1}). Soil from vineyard 2 contained significant levels of MET; thus, it was not fortified with this compound in the laboratory dissipation study.

4. Conclusions

SFC-ESI-QTOF-MS permitted the chiral, sensitive determination of five fungicides widely employed in viticulture and, in general, in agriculture. The modifier added to supercritical CO_2 was the only parameter showing a significant influence on the selectivity of chiral separations. On the other hand, additives played compound and mobile phase dependent effects in the yield of their ionization at the ESI source. Data obtained for processes samples (wines and soils) point out to the fact that vineyards are still treated with formulations including the very low active enantiomer of MET (S-form). Thus, without a record of vines treatments, through analysis of commercial wines is hard to investigate potential enantioselective removal of MET isomers during interaction with vines and/or through vinification steps. As regards vineyard soils, field data and laboratory experiments confirmed the enantioselective degradation of MET. The relative dissipation rates of R and S-forms differed significantly among soils from different vineyards. Despite BEN belongs to the same chemical family as MET, variations of its EFs during soil incubation assays were more subtle.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

L. Pérez-Mayán: Investigation, Methodology, Writing - review & editing. **M. Ramil:** Data curation, Formal analysis, Writing - review & editing. **R. Cela:** Project administration, Funding acquisition, Writing - review & editing. **I. Rodríguez:** Conceptualization, Funding acquisition, Funding acquisition, Writing - original draft.

Acknowledgments

L.P.M acknowledges a FPU grant to the Spanish Ministry of Science. This study was supported by Xunta de Galicia and Spanish Government through grants GRC-ED431C 2017/36, PGC2018-094613-B-I00, both co-funded by the EU FEDER program.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2021.462124.

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