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Evolution of fungicide residues in pruned vine-shoots

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ABSTRACT

Pruned vine-shoots used as wine additives have proven to be a useful tool for improving and differentiating wines. This is because they accumulate substances from the plant itself, which later, as a result of the toasting process, express their greatest oenological potential. However, vine being one of the crops subject to the most phytosanitary treatments, including fungicides in particular, it can be assumed that their residues will accumulate in the vine-shoots and pass into the wine. The aim of this study was to determine the content in pruned vine-shoots of four of the main fungicides applied in the vineyard in Spain: trifloxystrobin, boscalid, kresoxim-methyl and penconazole. In order to do so, a HPLC-MS/MS method was developed, which showed high reliability given its adequate validation parameters. The method was linear for the concentration range studied, LOD was 0.003 mg/kg and LOQ was 0.01 mg/kg, which comply with the control criteria.

The evolution of residues in vine-shoots complying with *critical agricultural practices* and *good agricultural practices* was monitored at 1, 3 and 6 months of storage after pruning and, in the latter case, after being subjected to a toasting process. The dissipation of all the residues was demonstrated to be affected by storage duration and toasting. It was confirmed that converting vine-shoots into oenological additives for wines will not pose a risk to the consumer if *good agricultural practices* are applied.

KEYWORDS

dissipation, oenological additive, fungicides, storage, toasted, vine-shoots

INTRODUCTION

Pruned vine-shoots are the most common waste produced in viticulture, with an estimated average world production of 1.95 x 10^7 tons per year. Nevertheless, due to its lower value, this waste is generally left or burnt in the vineyard, which generates environmental problems, such as the emission of greenhouse gases or soil erosion due to the loss of organic material (Peralbo-Molina and Luque de Castro, 2013).

However, the chemical composition of vine-shoots is characterized by some phenolic and aromatic compounds which have opened the possibility of using vine-shoots in viticultural and oenological practices. Specifically, aqueous extracts from vine-shoots have been proposed as vine biostimulants (Sánchez-Gómez *et al.*, 2016), since wines from vines sprayed with these extracts showed a *"feedback"* effect, with compounds from the vine being found in the wines, thus improving their chemical and sensorial profile. Recently, Cebrián-Tarancón *et al.* (2019) demonstrated the potential of using vine-shoots as oenological additives, due to their contribution to the aroma profile of wines when in contact with vine-shoot fragments. Vine-shoots from two varieties grown in the Castilla-La Mancha region (Spain), Cencibel and Airén,were stored for 6 months after pruning, granulated, toasted under specific conditions and added to wines at different times during winemaking. The herbaceous character of the resulting wines was significantly attenuated and the aromatic notes of dried fruits were increased. Moreover, in the different tasting tests, these wines were found to be of higher quality than the same wines that were not in any contact with vine-shoots (Cebrián-Tarancón *et al.,* 2019).

Due to the great interest in this new use for vine-shoots, it is necessary to assess the possible presence of pesticide residues, since pesticide and especially fungicide treatment on vineyard crops is widespread. To ensure that such wines do not compromise consumer safety, the presence of fungicides residues must be below the maximum residue limits (MRLs), or better still, totally exempted.

The control of pesticide residues, their evolution and transfer to plant material and food has been widely studied by several authors (Barba *et al.,* 1991; Marín *et al.,* 2003; Fernández *et al.,* 2005; Paya *et al.*, 2009), who analyzed the influence of different factors on the behavior of the pesticide residues. However, the authors did not find reference in the scientific literature to the presence of fungicide residues on vine-shoots. Consequently, the aim of this work was to determine the content of pesticide residues in vine-shoots when used as oenological additives. It was therefore necessary to develop a method for the extraction and analysis of several active fungicide materials, which were chosen from among the most commonly used in Spanish vineyards.

MATERIALS AND METHODS

1. Plant material: vine-shoots and treatments

Vine-shoots from two *vinifera* cultivars were used: one from the white variety Airén (VIVC: 157) and the other from the red Cencibel (VIVC: 12350) varieties, also known as Tempranillo Tinto. Vine-shoots were pruned in vineyards from O.D. Mancha (Castilla-La Mancha, Spain). Fifty vines in each vineyard were selected, and 0.5 kg of vine-shoots were pruned on each one; i.e., a total of 25 kg collected per variety and vineyard.

After that, they were grouped into 2 kg batches and divided into two groups: the first was stored intact (unfortified vine-shoots) and the second was sprayed with 200 mL of the different fungicides (fortified vine-shoots). After that, they were stored intact in the dark and at room temperature (18 ± 3 °C) for 1, 3 and 6 months, during which time the vine-shoots were subject to analysis. Each trial was performed in duplicate.

The active substances used were: trifloxystrobin, boscalid, kresoxim-methyl and penconazole, since these are the main fungicides used in the vineyards of the studied region. Table 1 shows the characteristics of these commercial fungicides.

The fortified vine-shoots were treated after pruning with a pool of active substances under *critical agricultural practices* at a dosage ten times higher than the legal limit. At the time of pruning, the vine-shoots did not have any leaves and the plant was dormant, so the fungicides could not be translocated.

The analyses of the fungicides were undertaken after treatment (Control) and after 1, 3 and 6 months of storage at room temperature of the intact vine-shoots. Moreover, a part of the vine-shoots which had been stored for 6 months was toasted. The unfortified vine-shoots, which were only in contact with fungicides during the vineyard treatments (considered as a *good agricultural practice)*, were analysed after 6 months of storage.

TABLE 1. Commercial fungicides characteristics and doses applied to vine-shoots under *critical agricultural practices.*

Commercial product	Active substances	Concentration	Form	Dosage
Flint	trifloxystrobin	50%	WG	13.75 g/hL
Collis	boscalid + kresoxim-methyl	20 + 10 %	SC	40 mL/hL
Topas	penconazole	10%	EC	35 mL/hL

WG: water granulate; SC: concentrated suspension; EC: concentrated emusable

For the analysis of fungicides, vine-shoots were pre-cut using pneumatic scissors then ground with a hammer mill (LARUS Impianti©, Skid Sinte 1000, Zamora, Spain) and sieved to a particle size of less than 10 mm.

2. Vine-shoot toasting procedure

The fortified vine-shoots stored for 6 months and then ground were toasted in an air circulation oven (Heraeus© T6, Hanau, Germany) at 180 °C for 45 minutes according to Cebrián-Tarancón *et al.* (2018). Such practice is necessary when using vine-shoots as an oenological additive in winemaking (Cebrián-Tarancón *et al.,* 2019).

3. Fungicide residue analysis

3.1. Extraction

Vine-shoot extractions were carried out according to the modified version of QuEChers method for the multiresidue analysis of grapes (Martínez *et al.,* 2015). 10 g of vine-shoot powder was shaken with 100 mL of acetonitrile for 1 min, then 4 g of magnesium sulphate, 1 g of sodium chloride, 1 g of sodium citrate dehydrate and 0.5 g sodium citrate sesquihydrate was added. This mixture was shaken vigorously for 1 min and centrifuged for 5 min at 3000 U/min. Finally, the separated extract was injected directly into the chromatograph after acidification with formic acid.

3.2. Analysis of fungicides by HPLC-MS/MS

The fungicide residue analysis was carried out according to Martínez *et al.* (2015), using an HPLC Agilent© 1200 with a DAD detector (Agilent©, Germany), which was coupled to a mass spectrometry system (ESI-MS/MS) with a triple quadruple 6410B (Agilent©, Germany) and equipped with a 120 EC-C18 Poroshell column (3.0 × 100 mm, 2.7 µm). Acetonitrile and formic acid (99:1, v/v) were used as solvent A and 0.1 % formic acid in ammonium formate 2 mM (0.1:99.9, v/v) as solvent B. The elution gradient started at 20 % of phase A (80 % B) and increased linearly to 100 % of A in 10 min - recovering the initial conditions in 2 min. The volume of the injected sample was 5 µL and the flow rate was 0.6 mL/min. Each sample was analysed in duplicate.

MS/MS detection was carried out in positive mode under the conditions established by Oliva *et al.* (2018). The capillary voltage was set at 3000 V, while the source and desolvation temperatures were kept at 120 and 350 °C respectively. A 1 l/min cone gas flow and 9 l/min desolvation gas flow were used. Multi-reaction monitoring (MRM) was used to detect all the compounds with a cycle time of 500 ms. All spectrometric parameters were individually optimised for each compound, as showed in Table 2.

In order to validate the method, we studied its linearity, precision (repeatability and reproducibility), recovery (accuracy) and the limits of quantification (LOQ) and detection (LOD). The LODs are the concentrations that produce an S/N of three, whereas the LOQs are based on an S/N of ten. Calibration curves of the compounds in the fungicide were made in triplicate at several concentrations (10, 20, 50, 100, 250, 500, and 1000 µg/L) using individual pure acetonitrile solutions of the standard fungicides.

TABLE 2. Experimental parameters and spectrometric conditions used for the identification of fungicides.

Fungicides	Precursor Ion, m/z	Quantifier transition, m/z	Qualifier transition, m/z	Fragmentor, V	Ce, V	t_R, min
boscalid	$[M+H]^+$ 343	343 → 307	343 → 140	130	20	7.57
penconazole	$[M+H]^+$ 284	284 → 70	284 →159	70	5	7.95
kresoxim-methyl	$[M+H]^+$ 314.1	314.1 → 206.1	314.1 → 267	80	5	8.53
trifloxystrobin	$[M+H]^+$ 409	409 → 186	409 → 206	70	5	9.43

TABLE 3. Calibration curves of vine-shoot fungicides (mg/kg).

Pesticide	Calibration curve	RSD (%)	R^2
boscalid	y = 0.298x + 0.003	8.59	0.9990
penconazole	y = 1.155x + 0.020	5.37	0.9999
kresoxim-methyl	y = 0.354x + 0.009	10.43	0.9998
trifloxystrobin	y = 5.498x + 0.066	5.65 5.65	0.9999

Concentration range (0.01-10 mg/kg)

TABLE 4. Recovery (%) and relative standard deviation (RSD) under the repeatability and reproducibility conditions of the studied fungicides.

Parameters	Recovery of repeatability				Recovery of reproducibility			
	%	% RSD	%	% RSD	%	% RSD	%	% RSD
mg/kg	LOQ: 0.01		10LOQ: 0.10		LOQ: 0.01		10LOQ: 0.10	
boscalid	100.2	2.6	106.0	2.5	101.8	3.1	103.2	4.0
penconazolee	96.0	4.0	108.7	2.8	99.0	4.5	107.2	3.1
kresoxim-methyl	101.7	3.1	110.0	3.0	100.3	1.6	106.2	4.2
trifloxystrobin	101.8	2.7	110.5	2.5	100.0	4.0	106.7	4.2

Ten replicates of Cencibel vine-shoots at LOQ (0.01 µg/kg) and 10LOQ (0.1 µg/kg) concentration levels were analysed under conditions of repeatability and reproducibility. 100 mL of the 100 µg/L solutions of the calibration curve were added to 10 g of toasted vine-shoot powder. Extraction was carried out as described in section 3.1. Repeatability was evaluated by assaying ten replicate samples at the same concentration during the same day, and with the same instrument and operator. Reproducibility was evaluated by assaying ten replicate samples at the same concentration, but on different days, and with the same instrument, but with different operators. Precision was considered to be satisfactory when RSD ≤ 20 % and recovery to be good with values of 70-110 %. Results were expressed in mg of active substance per kilogram of vine-shoot.

RESULTS

1. Validity of the method for analysing vine-shoot fungicides

Table 3 show the calibration curves of the vine-shoot fungicides, and that a good analytical linearity was obtained, with correlation coefficients (R^2) more or equal to 0.999 and a relative standard deviation (RSD) lower than 10.5 %.

The recovery results are shown in Table 4; in all cases the test results were acceptable, with recoveries ranging from 96 % to 100 % and RSD ≤ 4 %, which confirm that the method was sufficiently reliable for these four fungicides.

2. Evolution of vine-shoot fungicide residues during storage

The presence of fungicide residues in vine-shoots after pruning and 6 months of storage are shown in Table 5. The evolution of these residues in both vine-shoot varieties (Airén and Cencibel) was very similar, showing a considerable decrease in most of the fungicides with storage time. The higher residual value, 7.33 mg/kg, was observed in boscalid in the first fortified sample of the Cencibel analysis (Control); however, after 6 months its concentration decreased to 5.29 mg/kg. In both varieties, trifloxystrobin and kresoxim-methyl levels decreased after storage by about 40 % and 50 % respectively to below the maximum residue limit (MRL) established in grapes. MRL from the wine grape was used as a reference, as there is no data for fungicide in vine-shoots. On the other hand, penconazole decreased by approximately 30 % in both varieties, and boscalid decreased by nearly 25 %.

In unfortified vine-shoots, the concentrations of detected fungicides were insignificant; in all cases they were below the LOQ (Table 5).

TABLE 5. Evolution of fungicide residues in Airén and Cencibel vine-shoots during 6 months of storage.

Fungicides [mg/kg]	MRL	Airén					Cencibel				
		f				uf	f				uf
		Control	1 m	3 m	6 m	6 m	Control	1 m	3 m	6 m	6 m
boscalid	5	7.16 ± 0.11 d	6.26 ± 0.09 c	5.96 ± 0.05 b	5.38 ± 0.05 a	< LOQ	7.33 ± 0.05 D	6.33 ± 0.04 C	5.96 ± 0.08 B	5.29 ± 0.06 A	< LOQ
kresoxim-methyl	1.5	2.58 ± 0.08 d	2.21 ± 0.12 c	1.55 ± 0.10 b	1.25 ± 0.03 a	< LOQ	3.01 ± 0.06 D	2.52 ± 0.09 C	1.86 ± 0.11 B	1.40 ± 0.06 A	< LOQ
penconazole	0.5	3.60 ± 0.10 b	2.78 ± 0.09 a	2.68 ± 0.05 a	2.56 ± 0.05 a	< LOQ	3.92 ± 0.08 D	3.02 ± 0.06 C	2.82 ± 0.05 B	2.54 ± 0.05 A	< LOQ
trifloxystrobin	3	4.63 ± 0.07 d	3.32 ± 0.05 c	2.91 ± 0.06 b	2.53 ± 0.10 a	< LOQ	4.64 ± 0.05 D	3.49 ± 0.10 C	2.84 ± 0.07 B	2.64 ± 0.07 A	< LOQ

F = fortified vine-shoots, treated under *critical agricultural practices*; uf = unfortified vine-shoots, treated in vineyard under «good agricultural practices»; MRL = maximum permitted residue level in wine grapes (mg/kg). These data were taken from Regulation (EC) No. 396/2005, of the European Parliament of 23 February 2005, on maximum residue levels of pesticides in or food and feed of plant and animal origin. For each storage time, different lower case letters indicate significant differences between Airén samples and capital letters indicate significant differences between Cencibel ones, according to the Tuckey test ($p < 0.05$). The mean values ($n = 4$) are shown with their standard deviation.

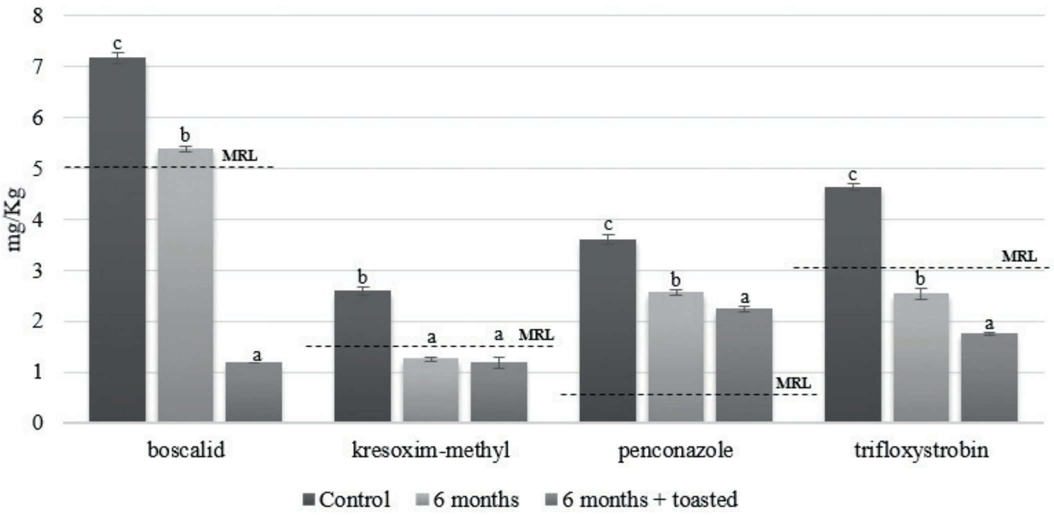

FIGURE 1. Effect of toasting on Airén vine-shoot fungicide residues.

Control = analysed immediately after treatment; 6 months = analysed after 6 months of storage after treatment; 6 months + toasted = analysed after 6 months of storage and toasting; MRL = maximum residue limit in wine grapes. For each substance, different letters indicate significant differences between sample times according to the Tuckey test ($p < 0.05$). The mean values ($n = 4$) are shown with their standard deviation.

3. Dissipation of fungicides on toasted vine-shoots

Fungicide dissipation in toasted vine-shoots to be used as oenological additives was studied. The toasting process is necessary for enhancing certain compounds which are important for wine, especially in relation to their aroma compounds. It is therefore necessary to study the presence of pesticide residues in the fortified vine-shoots after this heat treatment.

Figure 1 shows that when fortified Airén vine-shoots are toasted, the concentration of fungicides significantly decreases for some active substances, in contrast to the *critical agricultural practices* treatment (Control). The active substance boscalid was found to have the highest decrease in concentration by 84 %, a concentration of 1 mg/kg, which is five times lower than its MRL in wine grapes. Kresoxim-methyl levels decreased by 54 %, which was its initial concentration during storage, thus remaining constant with toasting, but with levels below the MRL. Compared to its initial concentrations, measured when vine-shoots were analysed after toasting, trifloxystrobin decreased by 62 %, thus also showing levels below the MRL in wine grape matrices. As regards penconazole, there was a 30 % decrease in its initial concentration with storage and a further 10 % with toasting. It must be taken into account that the vine-shoots had been fortified under *critical agricultural practices* (10 times more than the legal limit).

In Cencibel vine-shoots (Figure 2), the effect of toasting was more significant than in Airén. When vine-shoots were analysed after toasting, boscalid concentrations were found to have decreased by 90 % with respect to the Control (below 1 mg/kg). Kresoxim-methyl and trifloxystrobin decreased by 77 % and 74 % respectively in toasted vine-shoots with respect to the Control, and by around 50 % in both cases with respect to storage, with concentrations close to 1 mg/kg. Similar to Airén, penconazole was above the MRL permitted in grapes for this compound (0.5 mg/kg), but its initial concentration decreased by up to 35 % with storage and a further 20 % with toasting.

DISCUSSION

In recent years, several studies have shown that the chemical composition of vine-shoots comprises a large number of high-value oenological compounds. The use of vine-shoots has therefore been proposed as a new oenological additive for modulating the chemical composition and sensory profile of wines (Cebrián-Tarancón *et al.*, 2019).

It is well-known that the use of fungicide treatments in the grapevine agronomic cycle is common practice for the control of vine diseases. For this reason, the presence and evolution of their residues have been widely studied in grapes and wines (Barba *et al.*, 1991; Marín *et al.*, 2003; Fernández *et al.*, 2005; Paya *et al.*, 2009), but not in vine-shoots. However, if they are to be used

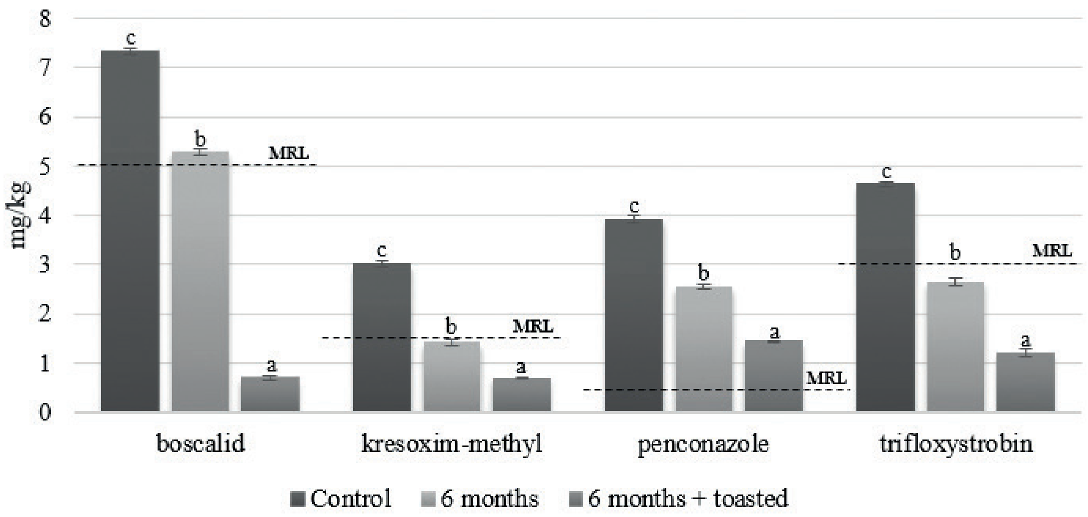

FIGURE 2. Effect of toasting on the Cencibel vine-shoot fungicide residues.

Control = analysed immediately after treatment; 6 months = analysed after 6 months of storage after treatment; 6 months + toasted = analysed after 6 months of storage and toasting; MRL = maximum residue limit in wine grapes. For each substance, different letters indicate significant differences between sample times according to the Tuckey test ($p < 0.05$). The mean values ($n = 4$) are shown with their standard deviation.

as a new oenological tool, exhaustive research is necessary.

It is important to note that this is the first study to focus on the evolution of fungicide residues in vine-shoots, which makes it difficult to compare the results with those of other studies from the literature. Other alternative oak wood products (chips and cubes, etc.) are used during the winemaking process, but the presence of these type of active substances has not been previously referenced. This makes it more complicated to compare our results with other wood oenological products used.

In this study, fungicide application was carried out after pruning; i.e., in winter when the vine was dormant and biological activity was less intense than during the growing season when treatments are carried out (leaves and grapes present). In some studies, some fungicides applied to grapes in the field during the summer period were found to have dissipated before harvest (Nadeem *et al.*, 2020; Yang *et al.*, 2020). In the present study, however, there was a higher concentration of the studied active substances (trifloxystrobin, boscalid, kresoxim-methyl and penconazole) in the vine-shoots to which they had been applied, since there was no translocation through the plant, and they could therefore not be distributed in order to be eliminated afterwards (as would have been the case if the treatments had been applied to the grapes). Moreover, under *good agricultural practices*, the last fungicide treatment is normally applied to vines during the summer months. However, as our study focused on the use of vine-shoots as an oenological additive, pruning was carried out in January, after which the vine-shoots were stored for 6 months and then toasted before use.

Once the active substances were isolated, the method was validated according to SANTE (2019) guidelines. The results obtained showed that the validated methodology was sufficiently reliable and accurate for the analysis of the fungicide residues in the vine-shoots. It was important to study the behaviour of the four active substances over storage time (1, 3 and 6 months). The fungicide treatments on the vine-shoots were carried out according to *critical agricultural practices* in terms of compound concentrations and application of active substances during plant dormancy.

The results showed a tendency for the fungicide residues to decrease over time, reaching their lowest levels after 6 months - the most reduced active substance being boscalid. This time point agrees with the one set by Cebrián-Tarancón *et al.* (2017), because it is when the highest content of high-value oenological compounds was reached. Therefore, if under these unfavourable conditions, vine-shoots did not show any residues from these fungicides, then they will likely show similar behaviour when treatment is applied in accordance with *good agricultural practices*.

Cebrián-Tarancón *et al.* (2017), also established that vine-shoots to be used as an oenological additive should be toasted; therefore, fungicide behaviour also needs to be monitored. While the post-pruning storage of 6 months clearly reduced the initial concentration of the active substances, the amount of residues was even lower when the vine-shoots were then subjected to a toasting process (Figures 1 and 2). Especially significant was the case of boscalid, whose concentration fell by up to 70 % in Cencibel and 60 % in Airén after toasting.

CONCLUSIONS

The results of this work confirm a decrease in the levels of boscalid, kresoxim-methyl, penconazole and trifloxystrobin residues in Airén and Cencibel vine-shoots from vineyards treated under *critical agricultural practices*, stored for 6 months and toasted. Therefore, these data indicate that the use of vine-shoots as an oenological additive for enhancing and differentiating wines is not likely to expose consumers to the four studied fungicides.

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