Glycoalkaloid Content in Pet Food by UPLC–Tandem Mass Spectrometry

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Abstract

The glycoalkaloid content of pet food containing potatoes is investigated using a liquid–liquid solvent extraction followed by analysis by ultra-high pressure liquid chromatography tandem mass spectrometry (UPLC–MS–MS). Pet food samples are homogenized and extracted with a solution of 50:50 (v/v) acetonitrile–deionized water containing 5% acetic acid. Following vortexing and centrifugation, 3 mL of the supernatant is filtered and diluted in deionized water. The extract is injected onto a reverse phase C18 UPLC column with an initial mobile phase composed of 0.15% acetic acid in water (A) and 0.15% acetic acid in methanol (B) in a ratio of 70:30, respectively. The mobile phase reaches a final concentration of 15% A and 85% B over 10 min, at which point it is returned to the initial conditions. α-Solanine is measured by monitoring transitions *m/z* = 868.50 → 398.40 and 868.50 → 722.50, while α-chaconine is measure by monitoring transitions *m/z* = 852.60 → 97.80 and 852.60 → 706.50. Each analyte is measured and combined to determine total glycoalkaloid content (TGA). The results of the analysis of 52 pet food samples indicate both glycoalkaloids are present in all samples and two pet foods were found to contain > 100 µg/g total glycoalkaloid.

Introduction

Potatoes belong to the Solanacea plant family, which is known to contain several toxic steroidal glycoalkaloids (GAs). These compounds act as a natural pesticide, protecting the plant against insects and fungi during growth and reproduction. α-Solanine and α-chaconine account for the majority of these compounds and are known to be toxic to animals and humans (1,2). α-Solanine and α-chaconine are nitrogen-containing steroids that vary structurally in their trisaccharide moiety (Figure 1). GA content is known to increase with exposure to sunlight or physical damage to the tuber with the higher content concentrated in the skin of the damaged part of the tuber and in the sprouts. Along with causing an increase in GA concentration, sunlight increases chlorophyll in the potato skin, greening the potatoes. This color change is an indication that the GA content may be high (3–6).

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Unintentional GA poisonings of humans and livestock have occurred with some cases resulting in severe illness and death (8,9,10). Because of this, all new potato varieties intended for human consumption are analyzed for TGA content and a generally accepted maximum safe level of 200 µg/g has been established. Due to the difficulty of toxicity estimation of GAs and diet assumptions, this guideline is still the subject of debate (2). Although GAs occur in all potato varieties, the amount and the ratio of the two compounds vary greatly depending on cultivar and the condition of the potato. Furthermore, because these compounds offer protection to the plant from potential damage, some amount of GA is considered a benefit to new varieties (7). Although the mode of toxic action in the body is the same for α-solanine and α-chaconine, the combination of the two generates a greater toxic effect than if either was present alone at the same concentration. This synergistic effect has been observed both in animal feeding studies and in antifungal activity (11–13). For this reason, an accurate, sensitive, and selective method for the

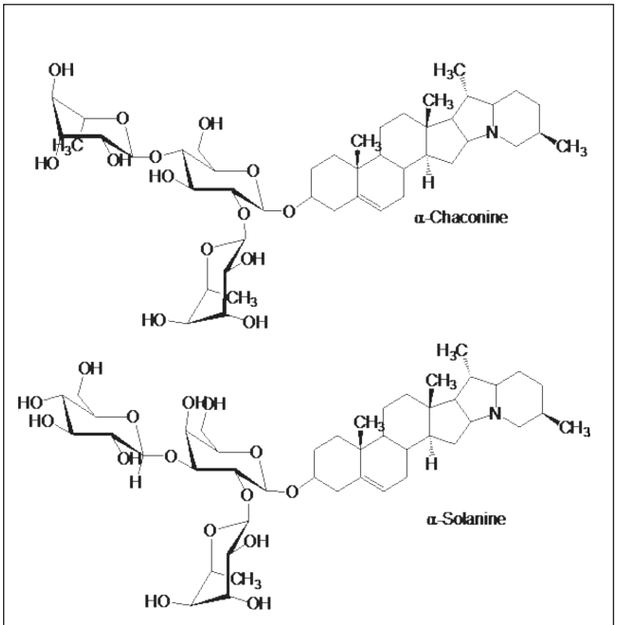

Figure 1. Structure of α-solanine and α-chaconine

measurement of α-solanine and α-chaconine in various foods and animal feed is necessary.

Various techniques have been used for the analysis of GAs in food including enzyme-linked immuno sorbent assay (ELISA), high-performance liquid chromatography (HPLC) with UV, and capillary electrophoresis (4,5,6,9,10,14). While ELISA is a rapid, low cost technique, it is limited to measuring total GA only and is unable to distinguish between α-solanine and α-chaconine. The most common technique is HPLC-UV, which allows for accurate quantitation of each GA; however, this method is susceptible to co-extracted matrix interference (3–5,9,10). Extensive sample clean-up is required and extraction efficiency losses are a risk if SPE is used. More recently HPLC–MS has been employed for GA analysis in food, which eliminates the need for extensive clean-up due to an inherent selectivity advantage over UV detection (7,15,16). Because HPLC–MS is a very sensitive technique, the final extract can be left fairly dilute and still achieve a detection limit in the ppb range. This level is more than adequate as GAs will usually be much higher in food products containing potato.

Most commercial dog and cat food contains products from many sources including potato waste from food manufacturers. Often this waste consists of the undesirable parts of the potato including skins and sprouts. Furthermore, if the skins are exposed to sunlight and the material is not fresh, sprouts will develop. These are the conditions under which GAs will become most highly concentrated (2,3,8,11). GAs in certain potato varieties have been shown to increase over 10 times when exposed to sunlight for 2 weeks (3). Fresh peels have been found to contain GAs as high as 868 µg/g and dehydrated peels as much as 3526 µg/g (2,7). Others have found that GA concentrations in sprouts can be over 7000 µg/g and leaves as much as 9000 µg/g (6,8).

A rapid, accurate method for the determination of GAs in wet and dry pet food was developed and applied to 52 samples.

Experimental

Reagents and chemicals

α-Solanine and α-chaconine were purchased from Sigma-Aldrich (St. Louis, MO). Methanol, acetonitrile, and acetic acid was purchased from J.T. Baker (Phillipsburg, NJ). Deionized water was generated with a Barnstead Nanopure water purifier with a conductivity of 18 mohm (Waltham, MA).

Stock solutions of 200 µg/mL α-solanine and 1000 µg/mL α-chaconine were obtained by diluting the appropriate amount of neat standard in methanol. A 10 µg/mL mixed standard containing both GA's was prepared from the stock solutions. To create calibration curves, standards of 0.001, 0.005, 0.01, 0.05, and 0.1 µg/mL in matrix were made by diluting the 10 µg/mL mixed standard with blank sample extract.

Sample preparation/extraction

A homogenous portion of each pet food sample was placed into a grinding mill (Retsch, Haan, Germany) and ground at a speed of 12000 rpm. The sample was ground to fineness using a 0.75 mm sieve. From the ground homogenous sample, 1 g was weighed into a 50-mL poly propylene disposable centrifuge tube (NUNC-Thermo-Fisher, Waltham MA), and 10 mL of 50:50 acetonitrile–distilled deionized water containing 5% acetic acid was added. The sample was shaken for approximately 1 min, vortexed (Thermo-Fisher, Waltham, MA) for 10 min, and centrifuged (Thermo-Fisher) at 2500 rpms for 10 min. An approximately 3 mL aliquot of supernatant was removed and filtered through a 0.45-µm filter (Whatman, Kent, UK) using a monoject lock syringe (Tyco Healthcare, Scaffhausen, Switzerland). The filtered extract was further diluted into an auto sampler vial using 250 µL of extract and 750 µL of distilled deionized water then vortexed. If the resulting extract appeared cloudy, the auto-sampler tube was centrifuged using a benchtop centrifuge (Thermo-Fisher) for 10 min, and the supernatant was transferred to another auto sampler vial.

UPLC–MS–MS

All samples were analyzed using a Waters Acquity UPLC (Milford, MA) coupled to a Waters Quattro Premier XE triple quadrupole mass spectrometer. The column was a BEH C18 1.7 µm, 1.0 × 100 mm UPLC with a Van Guard pre column to prolong the life of the analytical column. Mobile phase A consisted of 0.15% acetic acid in deionized water and mobile phase B was 0.15% acetic acid in methanol. The initial LC conditions were 70% A and 30% B and was held for 1.0 min. From 1.0 to 10.0 min, the gradient was changed from 70% A–30% B to 15% A–85% B. At this point the mobile phase was returned to the initial conditions and held for 3 min. The flow rate was held at 0.07 mL/min throughout the run. The injection volume was 10.0 µL, and the column temperature was 30.0°C.

The triple quadruple mass spectrometer was operated in multiple reaction monitoring (MRM) mode using ESI ionization. The desolvation temperature was held at 250°C, and the source temperature was 100°C. The desolvation gas flow was 900 L/h and the cone gas flow was 300 L/h. The MRM conditions for α-solanine and α-chaconine are described in Table I and were performed in positive ionization mode. The dwell time was 0.05 s for all transitions, which results in well over 50 scans across each peak.

For α-solanine the transition $m/z = 868.5 \rightarrow 722.5$ was used as the quantitation ion, and $m/z = 852.6 \rightarrow 706.5$ was used for α-chaconine. The signal for these transitions were plotted and integrated. The area counts were used for generation of standard calibration curves and for quantitation of spikes and samples. Analytes were identified by plotting the two transitions associated to each compound and comparing the retention time of the peaks to

Table I. MRM conditions for α-solanine and α-chaconine

				Product 1			Product 2		
	RT	MW	precursor	*m/z*	Cone V	collision energy	*m/z*	Cone V	collision energy
α-Solanine	8.20	868.1	868.5	398.4	80.0	75.00	722.5	80.00	70.00
α-Chaconine	8.10	851.0	852.6	97.8	120.0	80.00	706.5	120.00	70.00

those present in the standards. If both ions were present in a sample at a retention time within 5% of what was measured in the standard and the ion ratios were within 10% of what was measured in a standard, the analyte was determined to be identified.

Results and Discussion

The optimum MS conditions for each analyte were determined by infusing concentrated solutions of each compound directly into the mass spectrometer and adjusting parameters in real time through the tune page. Ten ppm standard solutions of α-solanine and α-chaconine were directly infused into the LC–MS source. A syringe pump was connected to the LC transfer line via a T, which carried the standard into the API ESI source. With the mass spectrometer in full scan mode, precursor ions of α-solanine and α-chaconine were determined to be *m/z* = 868.5 and 852.6, respectively (Figure 2). While isolating each precursor ion and monitoring fragmentation products, the collision energy was increased to a point where the product signal strength was maximized. Two product ions were identified for each analyte, which allows for ion ratio calculation, improving the quality of detection confirmation. The precursor ion of α-solanine *m/z* = 868.5 produces fragments of *m/z* = 398.4 and 772.5 while the α-chaconine precursor produces fragments of *m/z* = 97.8 and 706.5 (Figure 3). It should be noted that while *m/z* = 706.5 is produced as a fragment from both compounds, *m/z* = 722.5 was not seen in α-chaconine and therefore was chosen as the quantitation ion for α-solanine. This was done in order to improve selectivity between compounds and avoid compound crosstalk. Similar product ions have been observed even when an ion trap instrument is used (17). The conditions necessary for product ion production were used to generate an MRM method where both precursor product transitions were monitored for each analyte.

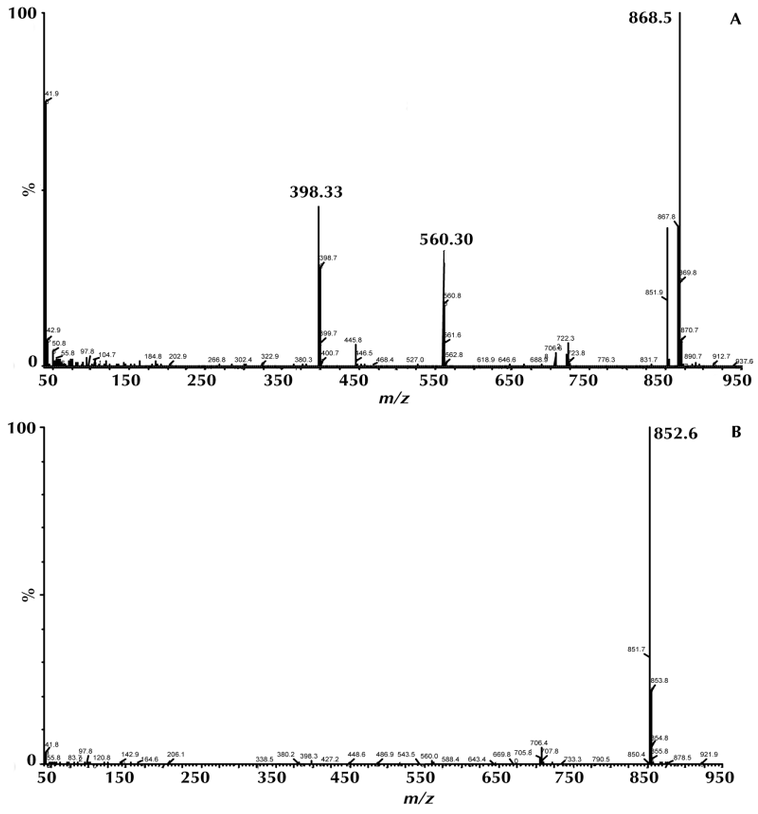

Figure 2. ESI positive ionization of a-solanine (A) and α-chaconine (B).

Table II. Recovery Efficiencies for α-Solanine and α-Chaconine in Wet and Dry Pet Foods

	Ave.% rec. (*n* = 5)			Overall ave.	Standard deviation	Correlation coefficient
	LOQ	5× LOQ	10× LOQ			
Solanine wet pet food	82.2	79.3	80.3	80.6	2.36	0.99991
Solanine dry pet food	84.7	108.5	100.6	97.9	11.5	0.99997
Chaconine wet pet food	92.5	81.1	83.7	85.8	8.14	0.99983
Chaconine dry pet food	99.9	97.7	91.3	96.3	8.42	0.99997

Wet and dry pet foods were obtained that did not contain potatoes for use as a matrix blank. Analysis of these samples revealed no detectable α-solanine or α-chaconine and no interference in the chromatographic region of the analytes. Extract of these blank samples was used as diluent for the production of various levels of standards. A mixed standard containing 10 µg/mL of both compounds was diluted to levels of 0.001, 0.005, 0.01, 0.05 and 0.10 µg/mL. α-Solanine and α-chaconine were found to elute at 7.65 min and 7.61 min, respectively. Although they elute very closely, their unique product ions provide sufficient selectivity to avoid misidentification.

Low level spiking experiments indicated that α-solanine and α-chaconine can be detected and confirmed at 30 ng/g and 20 ng/g respectively. Spikes at these levels were repeated twice in wet and dry food. The extracts were analyzed and compared with a matrix-matched standard at a similar concentration. The retention times for both analytes were found to agree to those of the spikes within 5% and all ion ratios matched those of the standard within 10%.

LOQ validation was accomplished by spiking blank wet and dry pet food samples at concentrations of 1×, 5×, and 10× the LOQ. Additionally each level spike was repeated 5 times in order to evaluate method repeatability. Recovery efficiency for both analytes was better in dry pet food, although recoveries for all analytes were between 80% and 100% (Table II). For wet pet food, a calibration curve was generated for each analyte from 0.001

µg/mL to 0.5 µg/mL using matrix matched standards. The curves provided linear response over this range with correlation coefficients of 0.9998 or better. Quantitation of 10× LOQ concentrations required an additional 10 fold dilution and the results indicated that samples in this range of concentration could be accurately quantified. For dry pet food standards from 0.001 µg/mL to 0.1 µg/mL were used to generate a calibration curve and correlation coefficients for these curves were 0.9999 or better. 52 dog and cat foods were identified for analysis due to the fact that potatoes were labeled as an ingredient. All samples were analyzed according the described procedure and any GA's that were reported met all conditions of confirmation. The results of analysis for α-solanine and α-chaconine are summarized in (Figure 4). The two samples found to contain TGA over 100 µg/g were both dry dog foods and were from different manufacturers. 5 samples contained TGA between 40 and 70 µg/g. Of these, two were dry cat foods from the same manufacturer, two were wet dog foods from the same manufacturer, and one was a dry dog food.

The sensitivity of the method is demonstrated in Figure 5. This sample was found to contain one of the lowest levels of GAs of all products analyzed. At a TGA of 0.19 µg/g this concentration was most likely well below any level of health concern however it illustrates the ability of the method to confirm and quantify these compounds even at low levels. For the 1.0 ng/mL standard the ratio of the transition 868.5 > 722.5 to 868.5 > 398.4 from α-solanine was 0.46 and that of sample # 50 was 0.48, well within the 10% acceptance window. The α-chaconine ratio of 852.6 > 706.5 was 0.27 for the 1.0 ng/mL standard and 0.29 for sample # 50 also well within the 10% acceptance window. Samples with higher analyte concentrations had generally better agreement between samples and standards and all reported results satisfied the acceptance criteria.

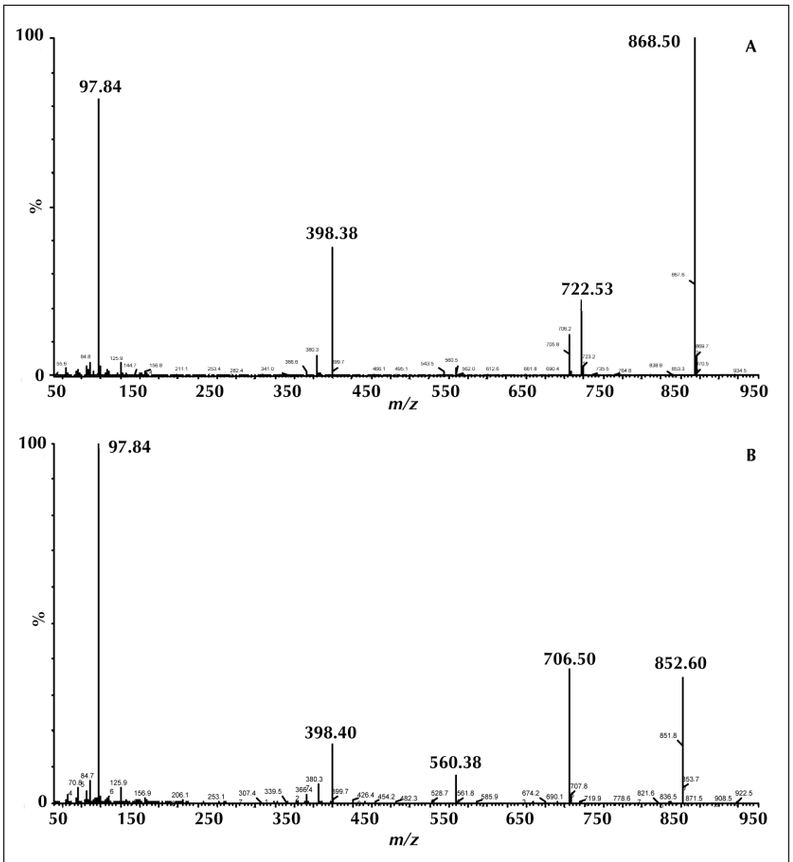

Figure 3. Product fragments of α-solanine (A) and α-chaconine (B).

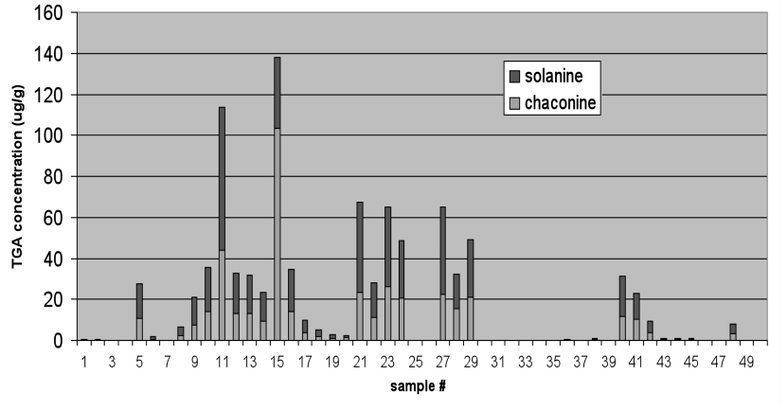

Figure 4. TGA concentration by sample number.

It is unclear at what concentration GAs become a health concern in food. The generally accepted guideline is for the TGA concentration in potatoes to be < 200 µg/g; however, this level has not been supported through toxicological studies and does not take into account the synergistic effects that have been shown to occur when α-solanine and α-chaconine are present in various proportions (2,12,13,18). Also, little is known of the possible chronic toxicity of GAs and no animal studies have been conducted to investigate this potential problem (8). In fact, very few animal studies have been conducted at all regarding the toxicity of GAs. In an investigation of experimental protein sources, rainbow trout fed a diet containing a GA content of 1 g/kg showed a significantly reduced growth rate; however, this concentration was quite high (19). A more relevant study involved the feeding of greened potatoes to rabbits. The concentration of GA in the greened potato group was 287.6 µg/g (ppm) whereas the GA concentration in the control group was 82.5 µg/g. The rabbits fed the higher GA potatoes suffered a decrease of 17.5% in their body weight and 20% of the animals died. Also, the rabbits fed high GA diets suffered from organ abnormalities including enlarged hearts and livers as well as hyperglycemia and other poor organ function (20). Considering the < 200 µg/g recommendation for potatoes takes into account the fact that the average human diet does not consist of potatoes alone, and the results of the rabbit study, a TGA limit for pet food would most likely be less than 200 µg/g. While no pet foods tested in this study

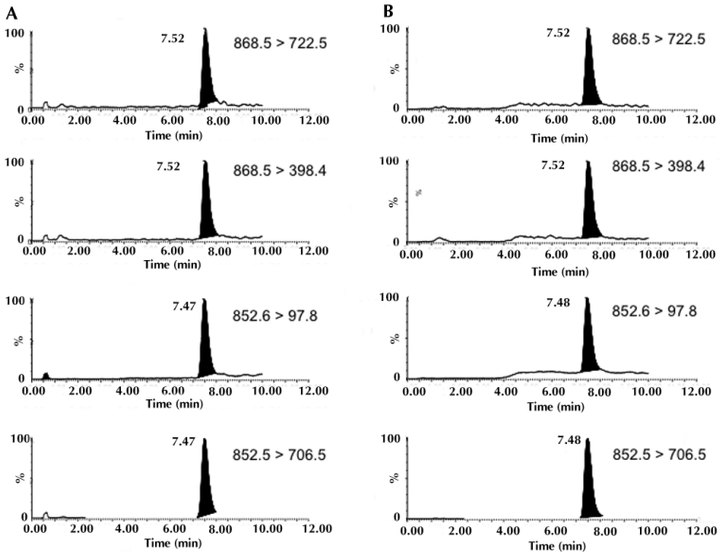

Figure 5. Chromatograms of precursor-product transitions for a 1.0 ng/mL of α-solanine (A) and α-chaconine (B). Sample # 49 containing 0.11 µg/g a-solanine and 0.088 µg/g α-chaconine.

indicated TGA levels greater than 200 µg/g, high levels in dog food have been found by this laboratory in the past. A dry dog food sample was analyzed for GAs after a dog was apparently made ill from the food. The dog suffered from digestive problems and analysis revealed a TGA concentration of 294 µg/g. It is possible that TGA concentration this high could be the cause of the illness.

Due to a lack of animal feeding studies, it is difficult to assess the danger of GAs in pet food. The recommendation that potatoes intended for human consumption contain TGA no higher than 200 µg/g is not applicable since pet food typically accounts for the great majority of the total diet as opposed to the small percentage potatoes account for in a human diet. Furthermore, it is possible that parts of the potato that would not be used for human food are being used in pet food. Unfourtunately, most of these parts such as greened peels and sprouts have a much higher GA content and may account for finished pet foods containing high TGA

Conclusion

The analysis of 52 pet foods containing potatoes found several products with TGA concentration over 50 ppm and 2 with TGA over 100 ppm. Since potatoes are not a primary ingredient in the product, the potatoes used must have had relatively high concentrations of TGA. This would suggest that greened potatoes, sprouts or varieties with naturally high TGA levels were used in the food production. While it is unclear at what level TGA can cause health risk in animals, the levels found in this study indicate possible concern. Since the majority of a pet diets consist of commercially sold pet food, the generally accepted warning limit of 200 ppm for potatoes intended for human consumption, may not be applicable for a warning limit in pet food.

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