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Tandem mass spectrometric methods for the analysis of iTRAQ labeled peptides in a quadrupole ion trap

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ABSTRACT

Alternative ion activation methods in a quadrupole ion trap mass spectrometer (QITMS) have been studied to determine their utility for iTRAQ. The collisional activation methods, thermally assisted CID (TA-CID) and high amplitude short time excitation (HASTE) CID, allow the low-mass cut-off (LMCO) to be reduced to ~10% of the parent ion mass-to-charge ratio allowing the iTRAQ reporter ions to be trapped and detected. An alternative to CID for ion activation/dissociation in a QITMS is infrared multiphoton photodissociation (IRMPD), which can be performed at LMCOs of <10% of the parent ion mass-to-charge ratio. Reported here are experiments comparing the efficiency of these methods for relative quantification using iTRAQ. All methods generated the reporter ions but there were differences in overall MS/MS efficiency and the conversion efficiency to the iTRAQ reporter ions.

1. Introduction

Stable isotope labeling is one approach to quantitative proteomics. Isobaric tag for relative and absolute quantification (iTRAQ) is one of the most common stable isotope labeling methods based on the covalent linkage of primary amines with tags with varying isotopic labels [1]. Quantitative information using iTRAQ is obtained from the abundance of low mass reporter ions, m/z 114–117 for a quadruplex reagent, observed in tandem mass spectrometry (MS/MS) spectra. Collision-induced dissociation (CID) is the most commonly used activation method for MS/MS. However, conventional resonance excitation CID in a quadrupole ion trap mass spectrometer (QITMS) occurs with instrumental conditions such that product ions below a LMCO of ~25% of the parent ion mass-to-charge are not trapped and detected. Thus iTRAQ reporter ions very rarely are detected in such an experiment. However, because of the widespread use of QITMS for proteomics experiments it would be valuable if iTRAQ experiments could be performed with these instruments.

The quadrupole ion trap (QITMS) is one of the most sensitive mass spectrometers, it has fast scan rates, and is especially efficient at MS/MS [2]. The vast majority of MS/MS experiments use CID. CID in a QITMS is performed using resonance excitation [3–6]. A supplementary radio frequency (rf) voltage matching an ion's secular frequency is applied to the end cap electrodes. The resonant condition increases the kinetic energy of the ion. A fraction of the kinetic energy is converted into internal energy via inelastic collisions with a helium bath gas until the critical energy of dissociation is reached and the ion dissociates to form product ions. A higher kinetic energy will result in greater internal energy gain, but it is possible that the supplementary voltage may increase the ion's kinetic energy so much that the ion is ejected from the trapping volume before it can dissociate. The amplitude of the supplementary rf voltage and the activation q_z of the ion must be carefully selected to achieve a sufficient kinetic energy, and concomitant internal energy gain, without ejection. A high q_z for activation, which is equivalent to a deep potential well, allows ions to be resonantly excited to high kinetic energy without exceeding the trap boundaries. A low activation q_z, i.e., shallow potential trapping well, often results in ions getting ejected from the trapping field prior to accumulating sufficient internal energy to dissociate.

From the previous statements, it would seem that CID should be performed at a high q_z, but Eq. (1) demonstrates why this is not so.

$$M_{\mathrm{LMCO}} = \frac{q_{\mathrm{P}}}{q_{ej}} M_{\mathrm{P}} \quad (1)$$

The mass-to-charge and q_z of the parent ion are represented by M_{P} and q_{P} respectively. The lowest mass-to-charge ion trapped corresponds to a q_z value of 0.908 (q_{ej}), termed the low-mass cut-off (LMCO), and is represented by M_{LMCO} in Eq. (1). It can be seen from this equation that a high q_z of activation (q_{P}) also means that a greater number of product ions fall below the LMCO and are not detected. To obtain effective dissociation and trapping along with a

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large mass range, CID is often performed at $q_z = 0.25$. At this q_z value product ions with mass-to-charge less than 27.5% of the parent ion's mass-to-charge are not stored; this is often called the "one-third rule" for quadrupole ion traps. Because the mass-to-charge ratio of the iTRAQ reporter ions typically is <20% of the parent ion mass-to-charge, when a QITMS is used the reporter ions are not trapped/detected. Thus, a QITMS using conventional CID cannot be used for iTRAQ experiments.

Other techniques have been developed for MS/MS in a QITMS that reduce the LMCO operating value. These methods include thermally assisted CID (TA-CID) [7], high amplitude short time excitation (HASTE) CID [8], and infrared multiphoton dissociation (IRMPD) [9–12].

TA-CID is implemented in exactly the same manner as conventional resonance excitation CID except that the He bath gas is heated above ambient temperature, typically to between 373 and 473 K. There are two positive affects with regard to CID that occur when the bath gas is heated. First, the parent ions have increased internal energy (equivalent to dozens of collisions with He in conventional resonance CID). Thus less additional internal energy is needed to reach the critical energy for dissociation. Therefore lower kinetic energies can be used to effect CID, which allows a lower LMCO to be used. The second advantage of TA-CID is that the product ions formed will collisionally cool slower, allowing more consecutive dissociation. Overall there is an increase in the absolute amount of parent ion dissociation and also an increase in the number of different product ions formed.

HASTE CID is also a resonance excitation process, but instead of using resonance excitation voltages in the 100 s of millivolt range and activation times of 20–40 ms, excitation voltages of 1–2 V are used with activation times of 1–2 ms. Then after the activation, the LMCO is quickly dropped to about 10% of the parent ion mass. The dissociation rate of ions in the QITMS is on the order of 100 s^{-1} so ions that dissociate on that time scale and are lower in mass-to-charge ratio can be trapped because the LMCO has been lowered. There is still the issue of competition between ejection and collisional activation in the HASTE CID experiment and because of this the MS/MS efficiencies are usually 50–75% lower than for conventional resonance excitation CID. This lower efficiency is the tradeoff to being able to trap and detect lower mass product ions. A similar approach to HASTE CID, called pulsed Q dissociation or PQD, has been demonstrated to work reasonably well for iTRAQ labeled peptides [13,14].

In contrast to CID, IRMPD is nominally independent of the QITMS trapping parameters so a quite low LMCO can be used for IRMPD experiments. However, the size of the ion cloud increases with decreasing LMCO, which decreases the overlap with the IR laser and decreases fragmentation efficiency [15]. In practice a LMCO between 5 and 10% is typically adequate to get good MS/MS efficiency. While the trapping parameters and hence the LMCO is not an issue with IRMPD, getting efficient dissociation is. Because the parent ions are not being kinetically excited, collisions with the He bath gas are much more efficient in removing internal energy from excited ions in IRMPD. Thus, in most cases supplemental ion activation or a reduced He pressure has to be used with IRMPD.

Because there are a number of advantages and disadvantages for each of the activation techniques, we have performed experiments to compare them for iTRAQ using ion trap mass spectrometry.

2. Experimental

2.1. iTRAQ labeling

Glu^1-fibrinopeptide B, EGVNDNEEGFFSAR, 1570.6 Da was labeled with iTRAQ 114 and iTRAQ 117 reagents (Applied Biosystems) using standard procedure. The iTRAQ label incorporation adds 144 Da to the peptide mass for a total mass of 1715 Da. All chemicals were obtained from Sigma–Aldrich Chemical Co. and used without further purification. The labeled peptides were dissolved in a 30:70:0.1% acetonitrile:water:trifluoroacetic acid mixture to a final concentration of 50 μM. As a proof of principle, iTRAQ 114- and 117-labeled peptides were mixed in 1:1, 1:2, 1:5 and 1:10 ratios for relative quantification. The doubly charged labeled peptide appeared at m/z 858 in the mass spectrum and was the parent ion for all experiments. Upon activation the reporter ions are generated in the product ion spectrum and relative quantification is done using peak height.

2.2. Mass spectrometry

Protonated peptides were generated using a in-house built nano-ESI source. Analysis was done with a highly customized Finnigan ion trap mass spectrometer (ITMS^{TM}) controlled by ion catcher mass spectrometer software (ICMS).[1] A helium bath gas pressure of 1.0×10^{-3} Torr was used for all experiments. Ion activation using conventional resonant excitation CID was performed at $q_z = 0.25$ for 30 ms. To perform TA-CID the helium bath gas was heated to 433 K using a Lesker 1000 W stab-in heater as previously described [7]. The parent ion was activated at a q_z of 0.1. Parent ion activation using HASTE CID was at the conventional q_z of 0.25 but a short activation time, 2 ms, was used. After activation the q_z was lowered to 0.1 in 10 μs to trap the low mass product ions. Single frequency excitation waveforms for activation were built externally to the ICMS software using a program written in LabVIEW®.

A 50 W Synrad CO_2 laser (10.6 μm wavelength) triggered by a TTL pulse from the ITMS^{TM} electronics was used for focused laser IRMPD [16] and TA-IRMPD. The beam path is directed through a ZnSe lens with a focal length of 38.1 cm, and 3.2 mm entrance and exit holes in the ring electrode. To align the laser the beam was translated axially until maximum dissociation was observed for a peptide. IR irradiation was at $q_z = 0.1$. The irradiation time for ambient temperature IRMPD was 10 ms and for TA-IRMPD it was 6 ms. TA-IRMPD was performed at a bath gas temperature of 85 °C.

MS/MS efficiency (Eq. (2)) and conversion efficiency (Eq. (3)) were used to compare all the methods studied.

$$E_{\mathrm{MS/MS}} = \frac{\sum D}{P_0} \tag{2}$$

$$E_{\mathrm{conversion}} = \frac{D_x}{P_0} \tag{3}$$

MS/MS efficiency is defined as the ratio of summed product ion abundance ($\sum D$) to the initial parent ion abundance before activation (P_0). Conversion efficiency measures the effectiveness of a method to generate the iTRAQ reporter ions [17]. The conversion efficiency is equal to the abundance of the product ion of interest, D_x (in this case the iTRAQ reporter ion) divided by the initial parent ion abundance before activation.

The MS/MS efficiency indicates the ability to dissociate the parent ion and trap the resulting product ions. In most cases the highest possible MS/MS efficiency is desired so the ion activation parameters are typically adjusted to maximize the MS/MS efficiency. A high conversion efficiency should provide better precision in the relative quantification measurement, but potentially at the expense of being able to determine the peptide sequence if that is not known.

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3. Results and discussion

3.1. Collisional activation

For the doubly charged iTRAQ labeled Glu^1-fibrinopeptide B parent ion the LMCO at the conventional q_z value of 0.25 is 236 Da using Eq. (1). At this LMCO only ions with mass-to-charge ratio of 236 or greater are trapped for subsequent mass analysis, therefore iTRAQ reporter ions were not detected (data not shown). Upon activation using TA-CID and HASTE CID the iTRAQ reporter ions are observed at m/z 114 and 117 (Fig. 1). The product ions formed by peptide backbone cleavages are labeled and can be used for peptide identification. In the HASTE CID spectrum, an ion at m/z 70 (asparagine, aspartic acid or arginine related mass ion) which corresponds to a parent ion q value of ~0.074 is observed. This product ion in the HASTE CID corresponds to a LMCO of 8% compared to 28% used in conventional CID.

3.2. Infrared multiphoton dissociation (IRMPD)

IRMPD differs from CID in that the absorption of multiple infrared photons causes an internal energy increase in lieu of increased kinetic energy from resonance excitation. The implication, in theory, is that ions can be activated at a low q_z value without causing ejection, which minimizes the LMCO issue affecting CID. In practice, collisional cooling, which is proportional to pressure and inversely proportional to temperature, competes with photoexcitation at the 1 mTorr operating pressure of the QITMS, leading to poor fragmentation efficiency [18,19]. For this reason, IRMPD in a QITMS is often done at low pressures. However, the reduced helium pressure results in poorer resolution and sensitivity [20]. Until recently, IRMPD experiments have been done with a collimated IR laser beam, not a focused beam. A practical way to reduce the collisional cooling effect while achieving optimum instrument performance is to focus the laser [16]. A focused laser allows maximum overlap of the laser beam waist with the ion cloud for improved fragmentation at shorter timescales. Fig. 2a is a focused laser IRMPD spectrum of iTRAQ114-labeled Glu^1-fibrinopeptide B. iTRAQ reporter ions are observed in high abundance relative to other product ions, for example, backbone cleavage ions. There are more backbone cleavage ions than in the CID spectra, probably due to consecutive dissociation, but in this case there is little, if any, additional sequence information to be obtained from these product ions.

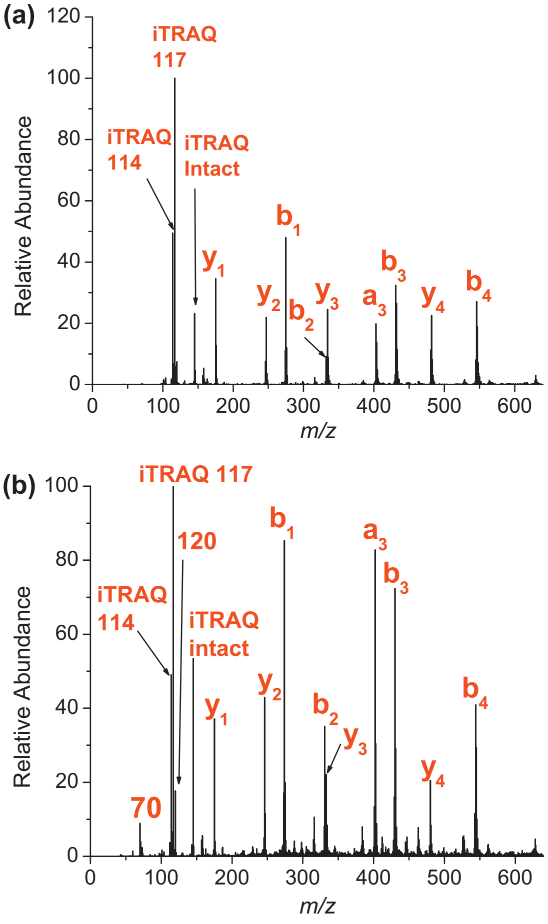

Fig. 1. MS/MS spectra of iTRAQ labeled Glu^1-fibrinopeptide B in a 1:2, 114:117 mixture using (a) TA-CID and (b) HASTE CID in a QITMS. Spectra shown for low mass region (0–640 Da).

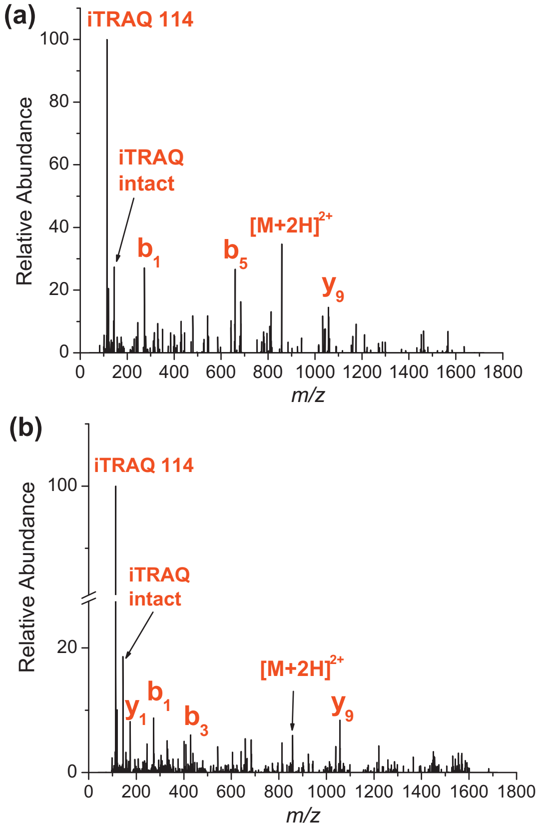

Fig. 2. MS/MS spectra of iTRAQ 114-labeled Glu^1-fibrinopeptide B using focused laser (a) IRMPD and (b) TA-IRMPD in a QITMS.

As an alternative to performing IRMPD at reduced operating pressures (i.e., <1 mTorr of helium gas required for optimum resolution and sensitivity) or focusing the laser, the helium bath gas can be heated. Analogous to TA-CID, thermally assisted IRMPD (TA-IRMPD) has been shown to be an efficient way to dissociate ions in a QITMS [11]. Just like TA-CID, heating the bath gas increases the parent ions internal energy prior to activation, thus require absorption of fewer photons. Also there is a slower rate of collisional cooling because of the smaller difference between the internal energy (temperature) of the activated ions and the temperature of the bath gas. Due to thermal activation prior to IR absorption, shorter activation times are used for TA-IRMPD compared to ambient temperature IRMPD. In Fig. 2b, extensive fragmentation is observed for 6 ms TA-IRMPD of iTRAQ114-labeled Glu^1-fibrinopeptide B. This time is 40% less than for the ambient IRMPD. Abundant iTRAQ reporter ion

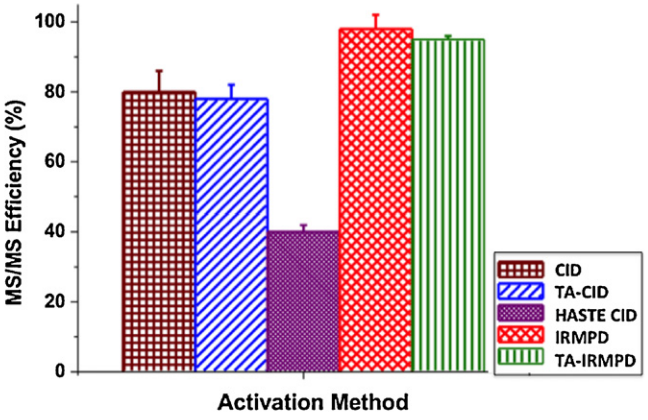

Fig. 3. MS/MS efficiency vs. ion activation method. Error bars are standard deviation of triplicate measurement.

signal is detected and even lower relative abundance compared to IRMPD was observed for peptide backbone cleavage product ions.

3.3. MS/MS and conversion efficiency

MS/MS efficiency is a measure of the extent of fragmentation achieved and the efficiency of collecting the product ions generated for mass analysis. Fig. 3 shows a plot of averaged triplicate measurement of MS/MS efficiency for all activation methods studied. MS/MS efficiency of TA-CID, although performed at a more shallow well depth, is comparable to that of conventional CID. The reason is that thermal activation causes parent ions to have higher internal energies before collisional activation. As a result lower supplementary rf voltages can be used to effect dissociation which allows lower q_z values to be used without ejecting the parent ions [21]. Lower q_z values allow the reporter product ions to be effectively trapped. HASTE CID resulted in the lowest MS/MS efficiency of the methods studied. The high amplitude excitation voltages needed likely cause the motion of some parent ions to exceed the boundaries of the trapping field leading to ion ejection and consequently a low MS/MS efficiency. Another possibility, although unlikely, is that the parent ion may dissociate to very small mass-to-charge product ions so that they are not detected even with the improved LMCO.

MS/MS efficiencies were close to 100% for both IRMPD techniques. In contrast to CID where the ions are kinetically excited and thus increase in amplitude of motion, the ions remain in the center of the QITMS during activation via IR photon absorption, so there should be no losses from ion ejection. The only losses that might be seen would result from an activation time of a length that consecutive dissociation occurs producing product ions below even the low LMCO used for IRMPD. Conversely, if the activation time used is not of sufficient length, inefficient fragmentation would result, also lowering the MS/MS efficiency. Fortunately, because there generally is little difference in the critical energies for dissociation of peptide, once the optimum length of the IR irradiation time is determined, that should work well for most peptides, scaled for peptide mass [22].

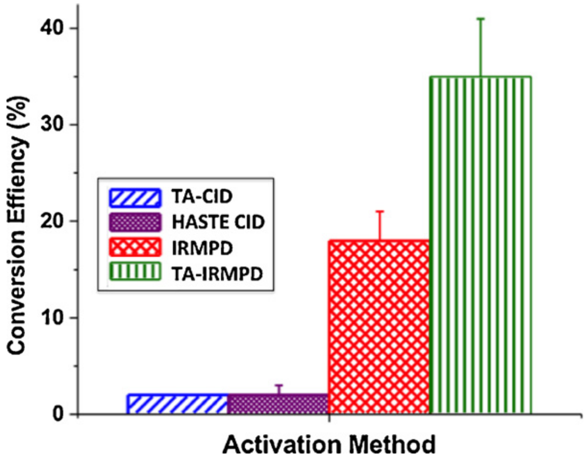

Fig. 4. Plot of conversion efficiency vs. activation method. Error bars are standard deviation of triplicate measurement.

Conversion efficiency measures the effectiveness of a method to generate the reporter ions. TA-CID and HASTE CID both gave conversion efficiencies of ∼2% as shown in Fig. 4. The likely explanation for the low conversion efficiency is that dissociation using CID is mostly a competitive process therefore many fragmentation pathways can be accessed as evident by the high abundance of backbone cleavage ions in the spectra. However, as discussed below, consecutive dissociation is limited because the product ions are not being activated, thus the a_n and b_n ions formed from the dissociation of the parent ion do not continue to dissociate down the fragmentation pathway to the iTRAQ reporter ions.

The IRMPD techniques give 10–20 times increase in conversion efficiency compared to the CID techniques. Consecutive dissociation is much more readily achieved when fragmentation is effected by IRMPD. For CID, the parent ion is resonantly excited and product ions formed rapidly undergo collisional cooling, minimizing consecutive dissociation [23]. In contrast, product ions formed by IRMPD are still in the path of the laser and thus can absorb photons and continue to dissociate. Thus, the a_n and b_n ions formed in IRMPD experiment, which still have the iTRAQ label, undergo successive dissociations with the terminal product ion being the iTRAQ reporter ion. The same a_n and b_n ions formed in the CID experiment will not be further activated and thus not dissociate to the simple iTRAQ reporter ion.

3.4. Quantification

While observing the iTRAQ reporter ions in the MS/MS spectrum is a requisite part of the experiment, to be useful the relative intensities have to correspond to the relative concentrations of the peptides. Relative quantification was examined by mixing the 114- and 117-labeled peptide at different ratios. Peak heights of triplicate measurements were averaged and the relative standard deviation was calculated. The results are shown in Table 1. All four techniques gave reasonable results in terms of accuracy and

Table 1
iTRAQ using TA-CID, HASTE CID, focused laser IRMPD and focused laser TA-IRMPD at various 114:117 ratios. Results are an average of triplicate measurements of reporter ion abundances.

Theoretical	TA-CID		HASTE CID		IRMPD		TA-IRMPD	
	Observed	RSD%	Observed	RSD%	Observed	RSD%	Observed	RSD%
1.0:1.0	1.0:1.0	8	1.0:1.0	4	1.0:1.0	4	1.0:1.2	7
1.0:2.0	1.0:2.0	3	1.0:1.9	4	1.0:2.0	0.5	1.0:2.1	1
1.0:5.0	1.0:6.3	18	1.0:5.5	10	1.0:5.8	2	1.0:6.8	2
1.0:10	1.0:24	27	1.0:17.2	14	1.0:12.0	7	1.0:13.1	4

precision when the relative amount of the peptides was similar (1:1 and 1:2). At a 1:5 ratio the techniques were similar in accuracy but the precision of the IRMPD methods was much better than that for the CID methods. At the highest ratio, 1:10, the CID techniques were much worse than the IRMPD technique for both accuracy and precision. The better results for IRMPD vs. CID at larger relative differences in peptide quantity probably are due to the much higher conversion efficiency obtained with IRMPD. The higher conversion efficiency means improved ion statistics, which should improve the precision, as observed, but will also probably affect the accuracy.

4. Conclusions

While relative quantification using iTRAQ typically cannot be performed using conventional CID techniques in a QITMS due to the low mass cut-off issue, alternative ion activation techniques can overcome this limitation for quantitative proteomics. Alternative CID techniques, TA-CID and HASTE, allow the reporter ions to be detected, but with low conversion efficiency. The MS/MS efficiency for TA-CID is similar to conventional CID while the HASTE CID efficiency is about 50% of the CID MS/MS efficiency, although still reasonable. The IRMPD techniques have much higher conversion efficiencies and also higher MS/MS efficiencies. This leads to better precision and accuracy for the IRMPD techniques vs. the CID techniques. These results suggest that a useful approach would be to do alternating scans of conventional resonance excitation CID and IRMPD analogous to the PQD and ETD experiment previously reported [14]. The CID MS/MS spectrum would provide the best sequence information while the IRMPD MS/MS spectrum would provide the best relative quantitative result.

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