

Research Article

Determination of 4-Methylimidazole in Ammonia Caramel Using Gas Chromatography–Tandem Mass Spectrometry (GC-MS/MS)

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One of Maillard reaction products formed in the production of ammonia caramel is 4(5)-methylimidazole (4-MeI) classified as carcinogen. A method of 4-MeI determination based on ion-pair extraction and derivatisation with isobutyl chloroformate with subsequent gas chromatography-tandem mass spectrometry analysis was proposed. Tandem mass spectrometry was applied to reduce the influence of matrix and increase the selectivity and sensitivity of the method. Triple quadrupole GC-MS system was used for this study. The collision energies were optimized for MRM mode. The detection (LOD) and quantification limits (LOQ) of the elaborated method were 17 and 37.8 $\mu\text{g kg}^{-1}$, respectively, repeatability was <15% RSD for analyzed caramel samples, and the recovery for 4-MeI was 101%. Comparison of MS/MS with SIM detection on the same instrument proved almost 30 times lower LODs achieved by tandem mass spectrometry compared to SIM. Described method can be routinely used for monitoring 4-MeI as a quality and safety marker in the production of ammonia caramel.

1. Introduction

Caramel is one of the worldwide most popular food colorants, labeled with the symbols E150a: caustic caramel, E150b: caustic sulfite caramel, E150c: ammonia caramel, and E150d: sulfite ammonia caramel. It is added for variety of foods and beverages, such as sauces, wines, beers, desserts, confectionary products, cola beverages, and baked goods [1]. One of many heterocyclic processing compounds formed in the production of ammonia caramel is 4(5)-methylimidazole (4-MeI), which is a product of Maillard reaction. At neutral to basic pH solutions there are two tautomers: 4-methylimidazole and 5-methylimidazole exist in equilibrium [2]. Methylglyoxal was proposed as the starting precursor in 4-MeI formation (Figure 1). In 2007 the National Toxicology Program (NTP) stated 4-methylimidazole as a cancer causing chemical and in 2011 the State of California also specified 4-methylimidazole in Proposition 65 as a carcinogen. The

level of 4-MeI, which is deemed to pose no significant risk, is 29 $\mu\text{g/day}$ [2].

The caramel matrix is very complex and it contains high number and amount of different compounds formed during caramelization process. One of those numerous components is 4-MeI. Its chemical properties, especially high polarity and basic character, make 4-MeI extraction and purification challenging steps. So far, analysis of this compound by GC/MS method was performed after ion pair extraction combined with derivatization process and this technique seemed to be the most effective one; however some recent studies showed the possibility of 4-MeI analysis by solid phase microextraction (SPME) method without derivatization [4]. Bis(2-ethylhexyl) phosphoric acid (BEHPA) is an ion pairing compound used in extraction of basic compounds and alkyl chloroformates (such as isobutyl chloroformate, IBCF) proved to be a good derivatization reagents providing formation of N-alkoxycarbonyl

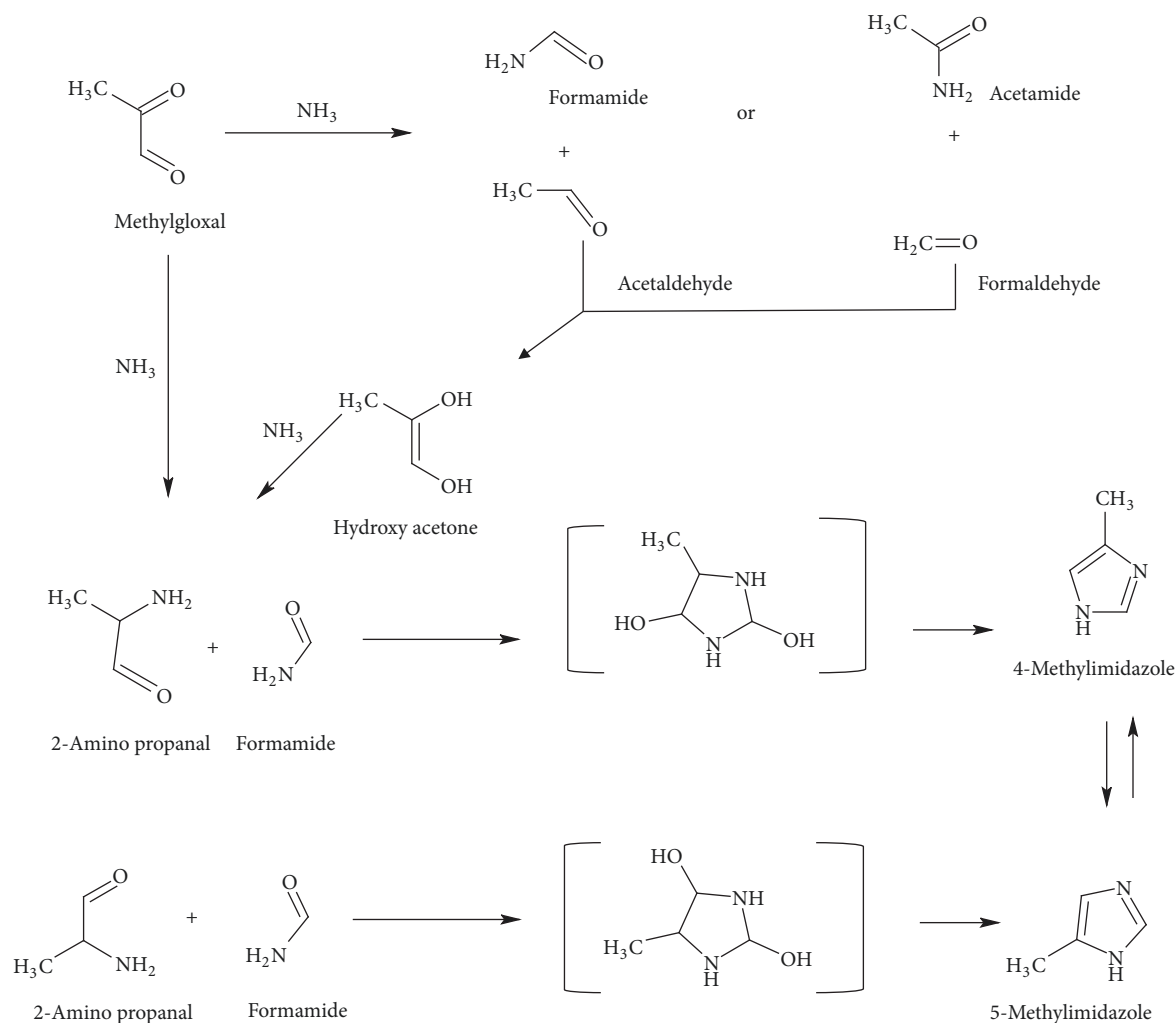


FIGURE 1: Proposed formation mechanisms of 4(5)-methylimidazole from methylglyoxal (adapted with permission from Moon and Shibamoto 2011 [3], Copyright (2011), American Chemical Society).

esters [5]. The Codex Alimentarius of the World Health Organization has established maximum concentration of 4-MeI in caramels III and IV, which is 250 mg kg^{-1} on an equivalent colour basis. It caused the necessity of developing and improving analytical extraction techniques, allowing for reliable detection of 4-MeI level in food and food additives matrices [6]. Described method of extraction was successfully applied for determination of 4-MeI in coffee [7]. Cunha et al. [8] used it to extract 4-MeI from soy and balsamic sauces and Karim and Smith [9] applied this procedure to determine the amount of 4-MeI in cooked meat.

The aim of this study was to develop a reliable and fast quantitation technique allowing for determination of 4-MeI in ammonia caramel produced on the pilot/industrial scale. The novelty of the study was to utilize the selectivity of tandem mass spectrometry for the qualitative and quantitative analysis, whereas usually GC-MS in SIM mode was used for 4-MeI analysis. By eliminating matrix influence in MS/MS (MRM) lower detection limits can be achieved.

2. Materials and Methods

2.1. Materials and Chemicals. Ammonia caramel (E150c) was produced in a pilot scale at the Institute of Agricultural and Food Biotechnology, Department of Food Concentrates and Starch Products, in Poznań.

4-methylimidazole (4-MeI), 2-ethylimidazole (2-EI), bis(2-ethylhexyl) phosphoric acid (BEHPA), isobutyl chloroformate (IBCF), hydrochloric acid, isooctane, and pyridine were obtained from Sigma-Aldrich, Poznań, Poland.

2.2. Sample Preparation and GC/MS Analysis. 4-Methylimidazole was extracted from caramel sample using methodology described earlier by Fernandes and Ferreira [5] and later by Cunha et al. [10]. The developed method is based on ion pair extraction with bis(2-ethylhexyl) phosphoric acid (BEHPA) and derivatization with isobutyl chloroformate (IBCF). The stock solutions of 4-MeI (100 mg L^{-1}) and the internal standard (2-EI, 1 g L^{-1}) were prepared by dissolving the compounds in 0.1 mol L^{-1} HCl.

The calibration curve was prepared by spiking caramel solution to compensate for matrix effect, with 4-MeI standard to obtain six concentrations: 0 $\mu\text{g mL}^{-1}$, 2 $\mu\text{g mL}^{-1}$, 4 $\mu\text{g mL}^{-1}$, 10 $\mu\text{g mL}^{-1}$, 20 $\mu\text{g mL}^{-1}$, and 40 $\mu\text{g mL}^{-1}$. Internal standard (2-EI) was added to all calibration solutions and to analyzed samples. Then, the analyte was extracted by ion pair extraction and derivatized according to described procedure. Extraction was done in triplicate for every concentration level. Additionally the calibration solutions were injected before the caramels analysis to check the repeatability.

2.2.1. Ion Pair Extraction. 3 grams of ammonia caramel was weighed and diluted with 10 mL of phosphate buffer and then potassium hydroxide was titrated to reach pH 6.0. Next, the whole mixture was transferred to 25 mL flask and filled up to 25 mL with water. 1 mL from this solution was transferred to the second vial and extracted with 2 mL 0.1 M bis(2-ethylhexyl) phosphoric acid (BEHPA) in chloroform. The mixture was mixed for 10 min. After centrifugation at 1500g for 10 min, the bottom layer (1.8 mL) was transferred to the third tube and then backextracted with 1.5 mL HCl. After centrifugation the upper layer was ready for derivatization.

2.2.2. Derivatization. A 500 μL aliquot of aqueous phase was mixed with equal amount (500 μL) of acetonitrile:isobutanol:pyridine (5:3:2 v/v) and 60 μL isobutyl chloroformate (IBCF). After 10 s of hand-shaking, 1 mL of saturated NaHCO_3 was added and sample was vortexed for 1 min. Afterwards, 1 mL of isooctane was added and sample was stirred for 10 min. Upon centrifugation the upper layer was ready for GC-MS/MS analysis.

2.3. Gas Chromatography-Tandem Mass Spectrometry. GC-MS/MS analysis was carried out on gas chromatograph coupled with triple quadrupole mass spectrometer (7890A/7000B, Agilent Technologies, Santa Clara, CA). Samples were injected in a splitless mode onto RTX-5MS column (10 m \times 0.18 mm \times 0.1 μm , Restek, Bellefonte, PA). The column oven temperature program during analysis was as follows: the initial temperature was from 70°C and maintained for 1 min, with temperature rise at a rate of 20°C min^{-1} , to 280°C. Temperatures of injector and mass spectrometer ion source were 280°C and 230°C, respectively. Both the analyzed compound and the internal standard were analyzed first in scan mode, then in SIM, and finally in MS/MS. The determination of product ions was accomplished in product ion mode, their origin checked in precursor ion mode, and multiple reaction monitoring (MRM) was used for quantitative purposes. The carrier gas was helium and the flow rate was 1 mL min^{-1} . Nitrogen was used as the collision gas and its flow was set to 1.5 mL min^{-1} , resulting in the highest areas of product ions. Collision energies tested varied from 5 to 50 V, which resulted in product ions of different intensities. The optimal collision induced dissociation (CID) value occurred at 10 V. In this process product ion spectra with a minimal precursor ion abundances and maximal of selected product ions were chosen for further method development. The mass spectrometer was operated in electron ionization (EI) mode

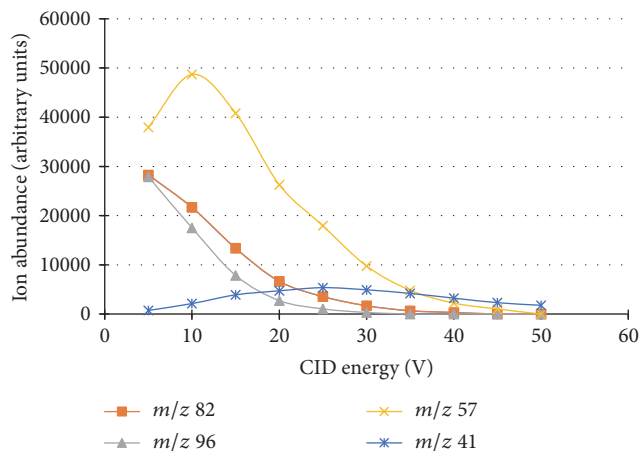


FIGURE 2: Effect of collision cell RF voltage (in a range of 10–50 V) on relative ion transfer efficiency of ions of different masses.

at 70 eV. Precursor ions used for MRM quantitative method were m/z 182 for 4-MeI and m/z 196 for 2-EI (internal standard). Precursor ions of both compounds were subjected to secondary fragmentation by which the resulting product ions were m/z 82 for 4-MeI and m/z 96 for 2-EI. Data acquisition and analyses were performed using the Agilent Technologies MassHunter Workstation software.

3. Result and Discussion

3.1. Gas Chromatography and Tandem Mass Spectrometry. In the present work the GC-MS/MS method was chosen to compensate for the complex matrix of Maillard reaction products that are formed in caramel production process. The selectivity was obtained by selective sample preparation involving specific extraction process and selective detection by MS/MS. The first step of the MS/MS optimization was the choice of the most selective and abundant precursor ion for 4-MeI derivative. The parent ion at m/z 182 was selected as the precursor ion for MS/MS analysis and m/z 82 as product ion. The m/z 82 ion was evaluated as the most specific one because 82 Da is the molecular mass of 4-methylimidazole. The intensity of m/z formed from fragmentation of m/z 182 was monitored in product ions mode using different CID energies (voltages) in a range of 5–50 V. 10 V was chosen for subsequent analyses as minimal abundance was obtained of ion m/z 182 with highly abundant product— m/z 82. Similarly, m/z 96 was used as a product of m/z 196 to be monitored in internal standard (4-EI), also indicating a molecular weight ion of this compound. The monitoring of most abundant product ions in 4-MeI and 4-EI is shown on Figure 2.

Linearity of the method was evaluated in the concentration range up to 40 mg L^{-1} ($\mu\text{g mL}^{-1}$). The concentration range was dependent on the expected concentration of 4-MeI in ammonium caramel. The coefficient of determination (R^2) was 0.99. The repeatability of the method when caramel samples were analyzed was satisfactory providing relative standard deviation values (RSD %) for samples run

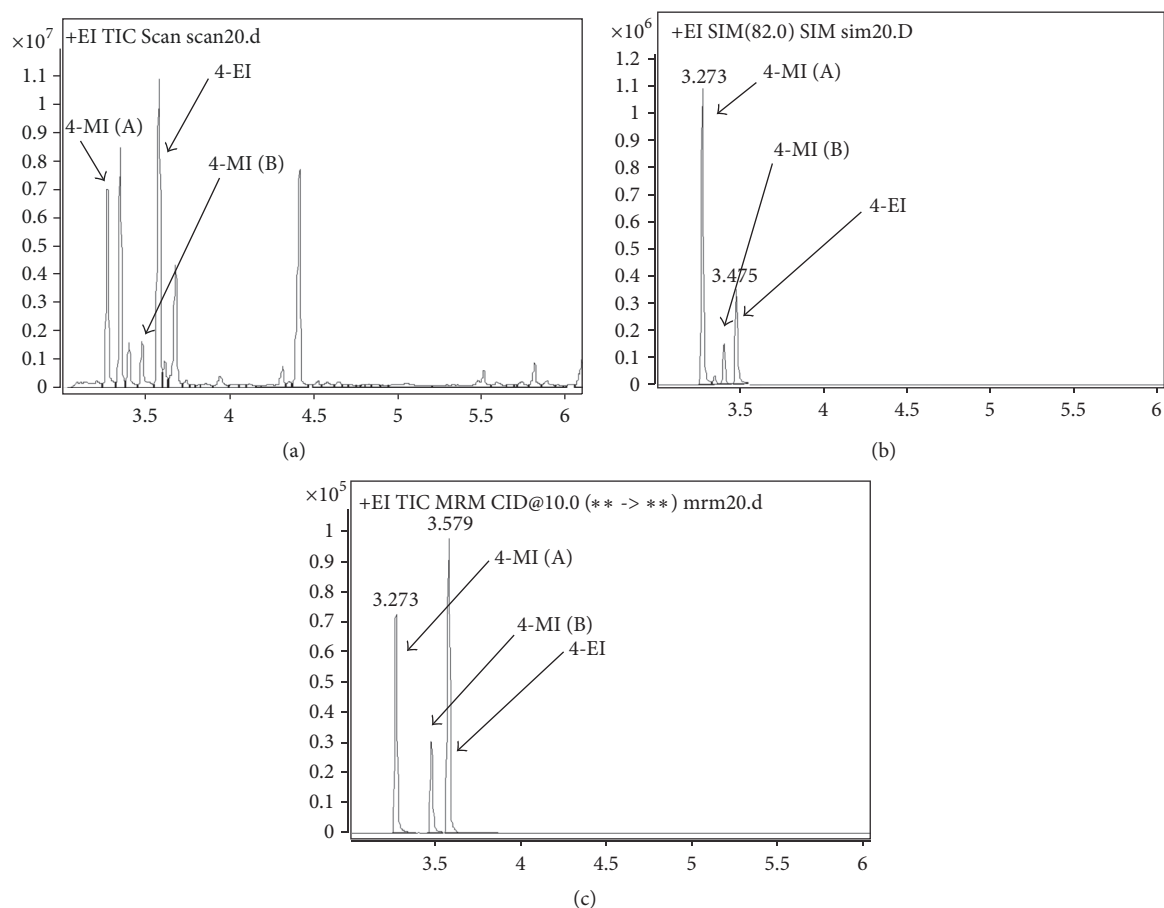


FIGURE 3: Chromatogram in SCAN (a), SIM mode (b), and MRM (c) of spiked caramel ammonia ($40 \mu\text{g mL}^{-1}$ of 4-MI (A and B) and $20 \mu\text{g mL}^{-1}$ of 2-EI (internal standard)).

in triplicate usually below 15%. No higher deviations were observed for intraday analyses. RSD values $<15\%$ were also observed with higher number of replicates of a single sample ($n = 7$). The recovery level of spiked sample was evaluated for one concentration ($40 \mu\text{g mL}^{-1}$) and it was 101%. In present work LOD and LOQ were calculated by using the signal-to-noise method. Noise before or after eluting peak was measured and, subsequently, the concentration of the analyte that would yield a signal equal to certain value of signal to noise ratio ($S/N = 3$ assumed in the work) is estimated [11]. LOQ was estimated as 2 LOD. In current work LOD and LOQ were $11.1 \mu\text{g kg}^{-1}$ and $24.7 \mu\text{g kg}^{-1}$, respectively, for MS/MS detection mode.

When 4-MeI is analyzed using ion pairing and IBCF derivatization method two peaks of the 4-MeI derivatives were obtained. They differed in retention times; however they share the same spectra with m/z 41 as the base peak resulting from fission of CH_3CN from imidazole ring, m/z 57 of all isobutyloxycarbonyl derivatives, and m/z 182 as the derivative molecular ion [5]. Chromatograms of ammonia caramel containing 4-MeI obtained in different operation mode of MS are shown on Figure 3.

As the method of 4-MeI in caramel and caramel containing food products require selective sample preparation,

ion pair extraction with subsequent derivatization with IBCF was used. The method of extraction and derivatization in GC/MS based applications offers the highest selectivity and has been used in different applications (Table 1). LOD and LOQ values remained close to these in GC/MS methods used by Fernandes and Ferreira [5], Casal et al. [7], and Cunha et al. [8, 10]. However, when experiments were run within this project on the same instrument (triple quadrupole) but ran in a single quadrupole SIM mode (first quadrupole set to transmission, collision gas switched off, and second quadrupole used for SIM) much higher LOD and LOQ values were obtained. Using the same method for S/N calculation, LOD and LOQ values in SIM mode were $338 \mu\text{g kg}^{-1}$ and $\text{LOQ} = 675 \mu\text{g kg}^{-1}$, respectively, which makes it almost 30 times higher than the values obtained for MS/MS method using the same instrument. Assuming the sensitivity of triple quadrupole instrument may be lower than that of a single quadrupole (longer ion path, need for ion beam focusing) obtained results illustrate the advantage of MRM over SIM detection.

Elaborated method was used to quantify the amounts of 4-MeI in 7 different samples of ammonia caramel produced. Results are shown in Table 2. The diversity of results obtained for analyzed samples of caramel reflects the differences

TABLE 1: Selected gas chromatographic methods used after extraction step, for the determination of 4-MeI in caramel colored food products.

Matrix	Extraction method	LOD	LOQ	References
Ammonia caramel	Ion-pair extraction	5 $\mu\text{g kg}^{-1}$	40 $\mu\text{g kg}^{-1}$	[5]
Roasted coffee	Ion-pair extraction	5 $\mu\text{g kg}^{-1}$	40 $\mu\text{g kg}^{-1}$	[7]
Soft drinks and dark beer	Ion-pair extraction	0.6 $\mu\text{g L}^{-1}$	2.2 $\mu\text{g L}^{-1}$	[10]
Cooked meat	Solution extraction	25 $\mu\text{g kg}^{-1}$	40 $\mu\text{g kg}^{-1}$	[9]
Balsamic vinegar and processed sauces	Ion-pair extraction	130 $\mu\text{g kg}^{-1}$	250 $\mu\text{g kg}^{-1}$	[8]
Ammonia caramel	Ion-pair extraction	11.1 $\mu\text{g kg}^{-1}$	24.7 $\mu\text{g kg}^{-1}$	<i>Current work</i>

TABLE 2: The content of 4-MeI in 7 different ammonia caramel samples produced in pilot scale.

Sample Number	Mean value [mg kg^{-1}]	SD	RSD [%]
1	11.1	1.56	14.0
2	6.47	0.34	5.25
3	13.4	0.44	3.39
4	8.34	0.89	10.7
5	12.3	1.51	12.3
6	10.4	1.10	10.6
7	10.4	1.61	15.6

SD: standard deviation; RSD: coefficient of variation.

in their manufacturing process. In all samples satisfactory reproducibility was achieved.

4. Conclusion

Described method, based on ion pairing and derivatization in sample preparation steps, followed by gas chromatography with tandem mass spectrometry was useful for determination of 4-MeI in highly complex matrix, which is ammonia caramel. As the matrix contains numerous compounds that may interfere with 4-MeI, selective extraction, derivatization, and detection were required. The novelty of this study was the use of MS/MS for quantitation of 4-MeI. As tandem mass spectrometry virtually eliminates the influence of matrix, especially coelution of ions of the same m/z , (as may happen in SIM), by their subsequent fragmentation, it allows more reliable quantitation and improved s/n ratio. The method proved to be more sensitive than SIM (determined on the same instrument, approximately 30 times) and was successfully applied to control levels of 4-MeI in ammonia caramel.

Disclosure

This work did not contain any studies with animal or human subjects.

Conflicts of Interest

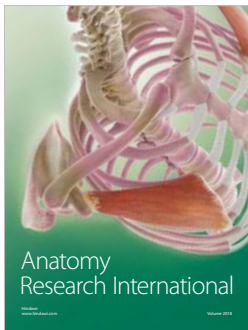
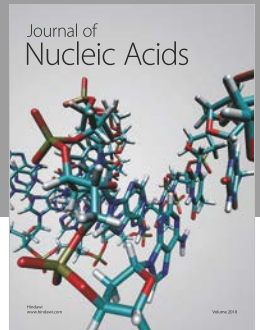
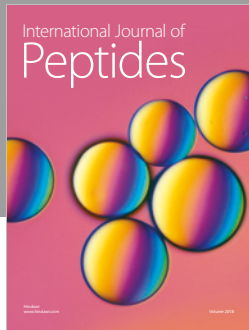
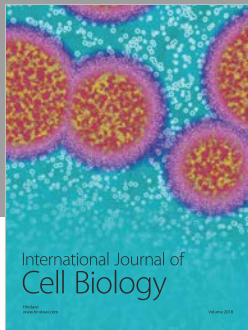
All the authors declare that they have no conflicts of interest.

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