Detection and Quantification of 4-Methylimidazole in Cola by Matrix-assisted Laser Desorption Ionization Mass Spectrometry with Fe$_2$O$_3$ Nanoparticles on Zeolite

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Food additives generally used in carbonated drinks, such as 4-methylimidazole (4MI), caffeine (Caf), citric acid (CA), and aspartame (Apm), were measured by matrix-assisted laser desorption ionization mass spectrometry (MALDI MS) using nanometer-sized particles of iron oxide (Fe$_2$O$_3$ NPs). The quantification of 4MI in Coca Cola (C-cola) was carried out. In order to improve the reproducibility of the peak intensities, Fe$_2$O$_3$ NPs loaded on ZSM5 zeolite were used as the matrix for quantification. By using 2-ethylimidazole (2EI) as the internal standard, the amount of 4MI in C-cola was determined to range from 88 to 65 μg/355 mL. The results agree with the published value (approx. 72 μg/355 mL). It was found that MALDI using Fe$_2$O$_3$ was applicable to the quantification of 4MI in C-cola.

Keywords MALDI, iron oxide, food additives, 4-methylimidazole, cola

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Introduction

Cola is a carbonated beverage that originally contained caffeine from kola nut and cocaine from coca leaves. Most colas today use other flavor ingredients with a similar taste and no longer contain cocaine. Cola gained worldwide popularity after pharmacist John Pemberton formulated Coca-Cola in 1886, and is now the most well-accepted beverage on Earth. The composition of cola is generally as follows: sweeteners (including glucose, fructose, and granulated sugar), condiment, caramel color, and caffeine. A can of Coke (355 mL) contains 39 grams of carbohydrates (all from sugar, approximately 10 teaspoons),† 50 mg of sodium, and 140 kilocalories.†

Cola has been criticized for its so-called deleterious effects on health.† For instance, a great many scientists have claimed that among the Cola additives, caramel color, which contains 4-methylimidazole (4MI), is the most harmful to human health. However, this claim has been questioned, and the debate on the safety of 4MI has thus continued to this day. 4MI is formed by the browning of certain foods through the Maillard reaction of carbohydrates and amino-containing compounds. In particular, it is found in roasted food, grilled meat, coffee, and certain types of coloring produced by an ammonia-based process.† Caffeine is also recognized as a harmful material. Excess caffeine intake causes high blood pressure and heart rate increase,§ and also hinders Fe absorption in the human body.¶ In contrast to colas, juice-type drinks are considered important non-carbonated drinks. The additives in juice-type drinks are generally citric acid, aspartame, sodium erythorbate, and so on. Citric acid is widely used as an emulsifier in the food industry, but excess intake may damage teeth¶ and other organs in the human body.¶ Aspartame is metabolized into methanol, aspartic acid, and phenylalanine. Phenylalanine, in particular, is thought to be dangerous to patients presenting with phenylketonuria,¶ and therefore the use of aspartame is usually indicated on juice labels.

The most widely used detection method for the additives in colas and juices is high-performance liquid chromatography (HPLC)† and synchronous fluorescence spectroscopy.† However, to the best of our knowledge, there is no report concerning additive measurements by matrix-assisted laser desorption ionization mass spectrometry (MALDI MS), although MALDI is advantageous for detecting molecules in many impurities. This is probably because MALDI has limited applications in the low-molecular-weight region because of the fragmentation of organic matrix molecules in the desorption ionization process. Instead of organic matrix molecules, the use of inorganic materials, particularly nanometer-sized metal or metal oxide particles, has been proposed.‡‡ There are several

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benefits of using nanometer-sized particles (NPs) as the matrix. First, fragment peaks of matrix do not appear during the laser desorption ionization process, thereby effectively extending the application of MALDI MS to low-molecular-weight compounds. Second, the mass spectrum of analyte becomes much simpler than that measured with conventional organic matrix molecules. Among NPs, iron oxide NPs (Fe₂O₃ NPs) are an effective matrix as well. For example, Chang et al. revealed that Fe₂O₃ NPs could be used for the extraction of peptides and proteins from a dilute solution through electrostatic attraction and hydrophobic interaction. Lin et al. synthesized functionalized Fe₂O₃ NPs and used them not only as a matrix, but also as a solid-phase extraction probe for small molecules. Ho et al. developed a seed-layer method and achieved homogeneous sample recrystallization, which yielded reproducible MALDI signals.

In this study, MALDI MS of soft-drink additives, including caffeine, 4MI, citric acid, and aspartame, was performed by using Fe₂O₃ NPs as the matrix. In addition, those compounds were also detected in a purchased cola sample. By using ZSM5 zeolite to improve the reproducibility of the peak intensity and 2-ethylimidazole (2EI) as the internal standard, the quantification of 4MI was carried out.

Experimental

Nanometer-sized particles of iron oxide (Fe₂O₃ NPs; >50 nm) were purchased from Sigma-Aldrich. Caffeine (Caf), 4-methylimidazole (4MI), 2-ethylimidazole (2EI), citric acid (CA), and aspartame (Apm) were purchased from Wako. Commercial Coca Cola (C-cola), Coca Cola Zero (C-cola zero), and Pepsi Cola (P-cola) were purchased from a supermarket. Proton-type ZSM5 zeolite (HZSM5) was supplied by the Catalytic Society of Japan (CSJ). Cola samples used for the study were poured into a beaker and degassed by ultrasonication to remove bubbles (CO₂) completely. The liquid without any bubbles was filtered through a 0.22-mm membrane filter and stored in a refrigerator for later use.

The inorganic matrix, Fe₂O₃ NPs, was dispersed in a mixture of ethanol and distilled water (4:1 in volume) using an ultrasonic bath, and the concentration was kept at 4 mg/mL. A solution of 2,4,6-trihydroxyacetophenone (THAP, 4 mg/mL) was also prepared. All of the analytes were dissolved in the mixed solution of ethanol and distilled water (4:1 in volume) and the concentration was kept at 1 mg/mL. One microliter each of the matrix and analyte was pipetted onto a sample plate, and the plate was exposed to air for several minutes to evaporate the solvent. Analyte ions were observed by using a commercial MALDI mass spectrometer (Waters, micro MS) equipped with a N₂ laser (337 nm, 10 Hz). A laser power of 6.0 μJ was employed for experiments using Fe₂O₃ NPs, whereas 8.0 μJ was used for THAP, and the beam diameter at the sample position was estimated to be <500 nm. Mass spectra were obtained by averaging 200 laser shots at random sample spots of whole area of a sample.

For the quantitative analysis, Fe₂O₃ NPs and HZSM5 in the weight ratio of 2:1 were mixed in a mortar and pestle, and 6 mg of the mixture was dispersed in the mixed solution of ethanol and distilled water (4:1 in volume). The obtained dispersion was used as the matrix solution. 2-Ethylimidazole at 0.2 μg/mL was prepared using distilled water. One microliter each of degassed cola (C-cola), 2EI, and matrix was pipetted onto a sample plate in that order. After the evaporation of solvent, MALDI MS was carried out.

Results and Discussion

Figure 1 shows the diffuse reflectance spectrum of Fe₂O₃ NPs. The spectrum of solid-state THAP is also shown for a comparison. Since the color of Fe₂O₃ NPs was almost black, the NPs had a broad absorption feature from the UV region to the near-infrared region. It can be seen from Fig. 1 that the absorbance of Fe₂O₃ NPs at the excitation wavelength for mass spectrometry (337 nm) was almost equal to that of THAP. Therefore, the different appearance of the mass spectra is due to the difference in the desorption and ionization properties of the two matrices. THAP was selected as a reference matrix for further MALDI MS studies since cola samples contain great amounts of sugars.

Figure 2(a) shows the mass spectrum of 4MI measured with THAP, an organic matrix molecule. The peak of protonated 4MI, [4MI+H]^+, was observed at m/z = 83. Analyte-related peaks were also observed at m/z = 105 ([4MI+Na]^+) and 121 ([4MI+K]^+). The mass spectrum of 4MI measured with Fe₂O₃ NPs is shown in Fig. 2(b). The above-mentioned three peaks of 4MI were observed, but their intensities were much higher than
those shown in Fig. 2(a). The intensities of [4MI+H]+, [4MI+Na]+, and [4MI+K]+ were ca. 17.2, 5.0, and 3.4, respectively. Notably, other noise peaks due to the matrix almost disappeared when Fe2O3 NPs were used. It is known that the disappearance of matrix-related peaks is one of the benefits of inorganic matrices. Therefore, Fe2O3 NPs are suitable as the matrix for the desorption ionization of 4MI.

Then, micrometer-sized Fe2O3 particles (Fe2O3 MPs hereinafter) were examined instead of Fe2O3 NPs, and the results are shown in Fig. 2(c). Three 4MI-related peaks were observed and their intensity ratio was almost the same as that of Fig. 2(b); however, the absolute intensities became smaller (approx. 1/4). As Fe2O3 MPs have a smaller surface area than Fe2O3 NPs, it is surmised that 4MI could not bind to the surface of Fe2O3 MPs efficiently.

Judging from the result that three 4MI-related peaks with almost the same intensity ratio can be observed in Figs. 2(b) and (c), it is considered that protons as well as sodium and potassium ions used for the ionization of 4MI were mainly provided by Fe2O3 particles. Sodium and potassium ions existed in Fe2O3 particles probably as impurities.

Caffeine (Caf), citric acid (CA), and aspartame (Apm) were examined next. Caf was subjected to MALDI MS using THAP as the matrix, and the result is shown in Fig. 3(a). Matrix peaks but not Caf-related ion peaks, were recognizable since the intensity of [Caf+H]+ was very low (ca. 0.3). When Fe2O3 NPs were used, the peak of [Caf+H]+ was observed at m/z = 195 at a high intensity (ca. 31.8), i.e., the peak intensity of [Caf+H]+ was increased by almost 106-times as shown in Fig. 3(b). Sodium-adducted Caf, [Caf+Na]+, was also observed at m/z = 217.

When MALDI MS was carried out for CA using THAP as the matrix, no protonated CA could be observed because of the acidity of CA (Fig. 3(c)). Instead, a sodium-adducted species, [CA+Na]+, was observed at m/z = 215, and its peak intensity was ca. 1.1. When Fe2O3 NPs were used, the peak intensity of sodium-adducted CA was ca. 9.2 as shown in Fig. 3(d). Potassium-adducted CA, [CA+K]+, was also observed at m/z = 231 and its peak intensity was ca. 3.3. In the same manner as Caf and CA, Apm could be detected efficiently by using Fe2O3 NPs instead of THAP, as shown in Figs. 3(e) and 3(f). Consequently, Fe2O3 NPs could be an efficient matrix for the desorption ionization of food additives, and we attempted to measure those food additives in colas next.

Figure 4 shows the mass spectra of Coca Cola (C-cola) measured with Fe2O3 NPs as the matrix. Figure 4(a) shows the spectrum in the mass range of m/z = 80 to 110. The peak of protonated 4MI was observed, although the origin of the strongest peak at m/z = 86 was unknown. In the quantitative analysis discussed in the next section, we used 2EI as the internal standard. We expected that the peak of protonated 2EI would appear at m/z = 97. Figure 4(a) shows that C-cola did not produce an interference peak at m/z = 97. Therefore, we considered it possible to use 2EI as the internal standard to quantify the amount of 4MI in C-cola, although the ionization and desorption efficiencies of 2EI must be different from those of 4MI. Figure 4(b) shows the mass spectrum in the mass range of m/z = 180 to 230. The peak of [Caf+H]+ was observed in this region. The most intense peak was that of potassium-adducted monosaccharide at m/z = 219. Another peak due to monosaccharide was measured as a sodium-adducted species at m/z = 203. The bottle label of C-cola indicates that high-
fructose corn syrup is used as an additive. As the sweetness of fructose, a monosaccharide, is the highest among sugars and its taste is enhanced at low temperature, high-fructose corn syrup is often used in soft drinks. In addition to Caf and monosaccharide, many origin-unknown peaks were observed. In Fig. 4(c), the peaks of sucrose, the major sugar component, were observed at $m/z = 365$ and 381. In this mass region, however, many origin-unknown high-intensity peaks were also observed.

In addition to C-Cola, Pepsi Cola (P-cola) and Coca Cola Zero (C-cola zero) were subjected to MALDI MS. Figure 5(a) shows the mass spectrum of P-Cola measured with Fe$_2$O$_3$ NPs. The additives confirmed in Fig. 4 were also observed in P-cola; however, other origin-unknown peaks were markedly reduced compared with C-Cola. Figure 5(b) shows the mass spectrum of C-cola zero. Instead of monosaccharides and sucrose, peaks of Apm were observed at $m/z = 317$ and 333 with strong intensities. In the same manner as Fig. 4, C-cola zero contained many kinds of additives whose origins were unknown. Therefore, it can be said that the kinds of additives used in P-cola are relatively less than C-cola.

Finally, a quantitative analysis of 4MI in C-cola was carried out by MALDI MS. It is widely known that MALDI is not suited for quantitative analysis because of low reproducibility. In our recent research, however, we found that zeolite improves the reproducibility of the peak intensities in MALDI MS, making MALDI MS applicable to quantitative analysis.\textsuperscript{26,27} In this study, we also used zeolite. Fe$_2$O$_3$ NPs loaded on HZSM5 were used in MALDI MS to determine the amount of 4MI in C-cola.

Prior to quantification, a regression curve of the peak intensity ratio and the amount ratio of 4MI to 2EI was generated, as shown in Fig. 6(a). To calculate the peak intensity ratio, peaks of protonated 4MI and 2EI were used. Measurements to calculate the peak intensity ratio for each amount ratio of 4MI/2EI were carried out three times for each of the three sample spots. The results are summarized in Table 1; overall

Table 1  Peak intensity ratios of \([4MI+H]^+/[2EI+H]^+\) for different amount ratios 4MI/2EI, and standard deviations

| Amount ratio of 4MI/2EI | Intensity ratio ([4MI+H]^+)/([2EI+H]^+) | Average | RSD, % | Overall average | Standard deviation | RSD, % |
|------------------------|----------------------------------------|---------|--------|-----------------|-------------------|--------|
| 0.25:1                 | Spot 1: 0.2415 0.2049 0.2176 0.2213 8.392 | Spot 2: 0.2222 3.408 | | Spot 3: 0.2337 0.0206 8.83 | 0.2337 0.02064 8.83 |
|                        | Spot 2: 0.2160 0.2306 0.2201 0.2222 4.008 | Spot 2: 0.2956 0.01278 4.32 | | Spot 3: 0.2923 0.03453 6.63 | 0.2923 0.03453 6.63 |
|                        | Spot 3: 0.2667 0.2466 0.2592 0.2575 3.934 | Spot 2: 0.2849 4.008 | | Spot 3: 0.2923 0.03453 4.32 | 0.2923 0.03453 4.32 |
|                        | Spot 1: 0.3110 0.2864 0.3318 0.3098 7.331 | Spot 2: 0.2851 4.008 | | Spot 3: 0.2851 4.008 | 0.2851 4.008 |
|                        | Spot 2: 0.2734 0.2962 0.2851 0.2849 3.934 | Spot 3: 0.2923 7.609 | | Spot 3: 0.2923 7.609 | 0.2923 7.609 |
|                        | Spot 3: 0.3161 0.2721 0.2887 0.2923 7.609 | | | | |
| 0.5:1                  | Spot 1: 0.5559 0.5466 0.5766 0.5597 2.751 | Spot 2: 0.5311 0.5106 7.201 | | Spot 3: 0.5211 0.03453 6.63 | 0.5211 0.03453 6.63 |
|                        | Spot 2: 0.3110 0.2864 0.3318 0.3098 7.331 | Spot 2: 0.5324 7.201 | | Spot 3: 0.5211 0.03453 6.63 | 0.5211 0.03453 6.63 |
|                        | Spot 3: 0.2667 0.2466 0.2592 0.2575 3.934 | Spot 3: 0.5324 7.201 | | Spot 3: 0.5211 0.03453 6.63 | 0.5211 0.03453 6.63 |
| 1:1                    | Spot 1: 0.5559 0.5466 0.5766 0.5597 2.751 | Spot 2: 0.5311 0.5106 7.201 | | Spot 3: 0.5211 0.03453 6.63 | 0.5211 0.03453 6.63 |
|                        | Spot 2: 0.3110 0.2864 0.3318 0.3098 7.331 | Spot 2: 0.5324 7.201 | | Spot 3: 0.5211 0.03453 6.63 | 0.5211 0.03453 6.63 |
|                        | Spot 3: 0.2667 0.2466 0.2592 0.2575 3.934 | Spot 3: 0.5324 7.201 | | Spot 3: 0.5211 0.03453 6.63 | 0.5211 0.03453 6.63 |
| 2:1                    | Spot 1: 0.6455 0.6548 0.6716 0.6573 2.013 | Spot 2: 0.6438 0.6196 5.433 | | Spot 3: 0.6232 0.05366 8.61 | 0.6232 0.05366 8.61 |
|                        | Spot 2: 0.6455 0.6548 0.6716 0.6573 2.013 | Spot 2: 0.6509 5.433 | | Spot 3: 0.6232 0.05366 8.61 | 0.6232 0.05366 8.61 |
|                        | Spot 3: 0.5191 0.6181 0.5468 0.5613 9.095 | Spot 3: 0.6509 5.433 | | Spot 3: 0.6232 0.05366 8.61 | 0.6232 0.05366 8.61 |
| 4:1                    | Spot 1: 0.8010 0.8447 0.7815 0.8090 3.999 | Spot 2: 0.7326 4.140 | | Spot 3: 0.74412 0.06001 8.07 | 0.74412 0.06001 8.07 |
|                        | Spot 2: 0.7588 0.6994 0.7397 0.7326 4.140 | Spot 2: 0.7326 4.140 | | Spot 3: 0.74412 0.06001 8.07 | 0.74412 0.06001 8.07 |
|                        | Spot 3: 0.7074 0.6977 0.6670 0.6907 3.058 | Spot 2: 0.7326 4.140 | | Spot 3: 0.74412 0.06001 8.07 | 0.74412 0.06001 8.07 |

Fig. 5 MALDI MS spectra of (a) Pepsi Cola (P-cola) and (b) Coca Cola Zero (C-cola zero).

Fig. 6 (a) Correlation between the peak intensity ratio ([4MI+H]^+)/([2EI+H]^+) and the amount ratio (4MI/2EI). (b) MALDI MS spectrum of C-cola with 2EI.
average, standard deviation (SD), and relative standard deviation (RSD) were calculated for each amount ratio. RSD values when MALDI MS was carried out without zeolite support (only Fe₂O₃ NPs were used as the matrix) are indicated in parentheses. It was confirmed that the RSD values were decreased and the reproducibility was improved when zeolite was used. Plots of the peak intensity ratio vs. amount ratio were fit by a single exponential function, and the fitting curve could reproduce the results well. As mentioned in Fig. 4, the ionization and desorption efficiencies of 4MI and 2EI are different molecules. In fact, the desorption efficiencies of 4MI and 2EI should be different.

Conclusions

Food additives, such as 4-methylimidazole (4MI), caffeine (Caf), citric acid (CA), and aspartame (Apm), were measured by matrix-assisted laser desorption ionization mass spectrometry (MALDI MS) by using nanometer-sized particles of iron oxide (Fe₂O₃ NPs). It was found that Fe₂O₃ NPs could ionize those additives efficiently compared to a conventional organic matrix (THAP). The above-mentioned food additives in colas could be measured by using Fe₂O₃ NPs, although many origin-unknown peaks were observed at the same time. Then, the quantification of 4MI in Coca Cola (C-cola) was carried out. In order to improve the reproducibility of the peak intensities in the mass spectra, Fe₂O₃ NPs loaded on HZSM5 were used. By using 2-ethylimidazole (2EI) as the internal standard, the amount of 4MI in C-cola was determined to be 88 to 65 μg/355 mL. The results matched the published value (approx. 72 μg/355 mL), and it was confirmed that MALDI using Fe₂O₃ is applicable to the quantification of additives in soft drinks.

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