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Application Note

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Doping Tests

Development of Drug Testing by Direct Analysis of Equine Hair Using Imaging Mass Microscopy with iMScope *TRIO*™

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Doping inspection

The photo was provided by the CEO of Equine Racing Co., Ltd., Mr. Masaru Sese

1. Introduction

In the field of forensics, hair is currently attracting attention along with urine for drug test samples. The reason for this is that, generally, drugs have to be detected as metabolites in urine, so detection is not feasible if no urine sample can be collected while they are being eliminated. On the other hand, drugs in hair are absorbed into the hair without being metabolized and stay there for a long time. That is to say, while with urine the drug may be lost by being metabolized and excreted within a few days after the last intake, hair has the characteristic that the intake history is preserved in it for the long term, provided the hair is not cut.

Currently, the gas chromatograph mass spectrometer (GC-MS) and liquid chromatographic mass spectrometer (LC-MS) are the common means that have been put into practical use as the new measurement methods for these test samples. After washing and drying, the collected hair is cut to a length of about 5 mm to 1 cm, and analyzed through a process of extracting the drug from each of the cut hairs and purifying it. Human hair grows at a rate of 1 cm per month on average, so if the position of a hair sample where the detection was made can be identified, we can determine "when", "what kind of drug" and "how much" was in circulation. Note that literature by Ono, Mizuno et al. serves as reference for hair analysis in the field of forensic medicine, including the sample pretreatment method described above [(1)-(3)].

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This kind of hair analysis is currently engendering a great deal of expectation in connection with doping tests not just on humans but on racehorses too [(4), (5)]. The test samples for the equine hair analyses that have been reported to date have been from the horse's mane (hereafter "equine hair"). However, equine hair is generally long, and adequate washing and drying is required to remove contamination on the surface of the sample. Also, since the number of samples after cutting is large, it is easy to imagine that the pretreatment is very troublesome.

With this as the background, methods using mass spectrometry imagining (MSI) have been reported as new methods for hair analysis, rather than GC-MS or LC-MS. With MSI, the pre-treated samples can be analyzed directly. In recent years, a pioneering report on MSI for the detection of drug intake history in human hair has been produced by Kamata et al. [(6), (7)]. To detect the history with MSI, the cuticle has to be removed in the longitudinal direction, exposing the hair medulla. This process is difficult, so a dedicated device is being manufactured as described in reference 6, but even when using this device it is not possible to remove the cuticle on a length of more than about 1 to 2 cm. Unlike human hair, equine hair is very long, which makes this process even more troublesome, hence no examples of drug intake detection in equine hair have been reported to date. In this paper, we will introduce as an example the detection of dexamethasone phosphate, which is a steroidal anti-inflammatory drug, by performing MSI on 4 cm of equine hair, albeit with manual removal of the cuticle.

2. Mass Spectrometry Imaging

In mass spectrometry, molecules are ionized and their mass (actually a dimensionless quantity known as *m/z* obtained by dividing the mass by the valence of the ion) is measured based on the difference between the movement in an electric field and magnetic field. As already mentioned, MSI differs from existing methods using GC-MS and LC-MS in that the spatial distribution information can be visualized by analyzing the sample surface as is, without extracting the drug to be analyzed.

The usual practice for experiments is to prepare a sample section and rest it on a transparent plate that has been given electrical conductivity by, for example, coating it with indium tin oxide (ITO). Then it is ionized and subjected to mass spectrometry. When an analysis is performed, the area to be measured and the interval between measurement points are decided, and the mass spectrum for each measurement point is obtained together with position information. After the mass spectra for all the measurement points have been acquired, the *m/z* corresponding to the targeted molecule is selected from the mass spectrum, and the localization information on the targeted molecule is acquired from the intensity distribution of that mass spectrum. Therefore, unlike conventional imaging techniques, labeling such as immunochemical staining or GFP labeling is not required. Other characteristics include that parent compounds and their metabolites can be distinguished because molecules are detected directly, and that localization information on a variety of molecules can be obtained in a single analysis since numerous compounds can be ionized simultaneously and are subjected to the analysis.

3. Development Concept for iMScope *TRIO*

Currently, MSI can be performed on a variety of mass spectrometers. It is also possible to select a variety of ion sources, and there are also various mass separation units. Since 2004, the author has been involved in the development of the iMScope *TRIO* ™ imaging mass microscope in collaboration with Shimadzu Corporation [8], and is now developing various application research at the Osaka University Shimadzu Analytical Innovation Research Laboratory [9].

The development concept for this iMScope *TRIO* is shown in Fig. 1. Although it is possible to see structure with a normal microscope, it is difficult to acquire information on the various components. On the other hand, the iMScope *TRIO* performs mass spectrometry using matrix-assisted laser desorption/ionization (MALDI) in combination with microscopic observation of the sample. Ions generated on the sample surface are drawn in under a vacuum so they can be detected by mass spectrometry. By mapping the intensity distribution from the resulting mass spectrum, component distribution information with a high spatial resolution can be observed under a microscope. Because iMScope *TRIO* does not "see" the sample with visible light or radiation, collectively known as "electromagnetic waves", it can be called a "new eye" available to mankind.

Details on the iMScope *TRIO* are given in the following sections.

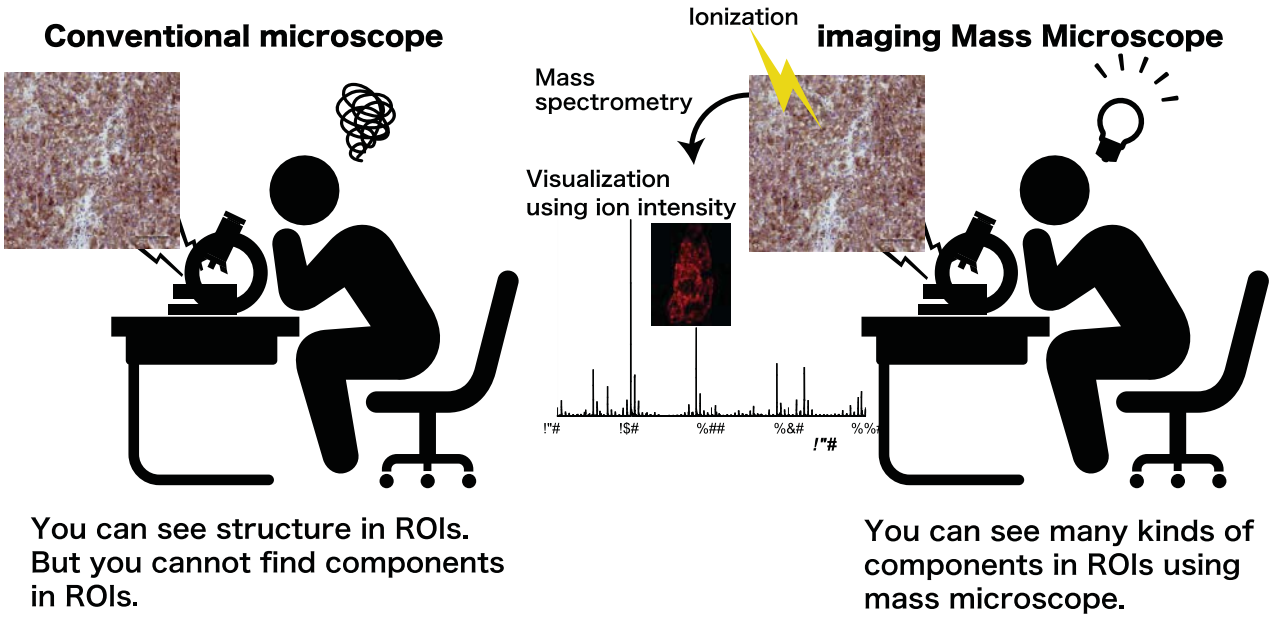


Fig. 1 **Concept for the iMScope *TRIO* ™ Imaging Mass Microscope**

With conventional microscopes, differences in structure can be determined, but it is difficult to acquire information about components. In contrast, the iMScope *TRIO* ™ performs mass spectrometry together with visual observation, so the intensity distribution can be obtained from the component information in the mass spectrum.

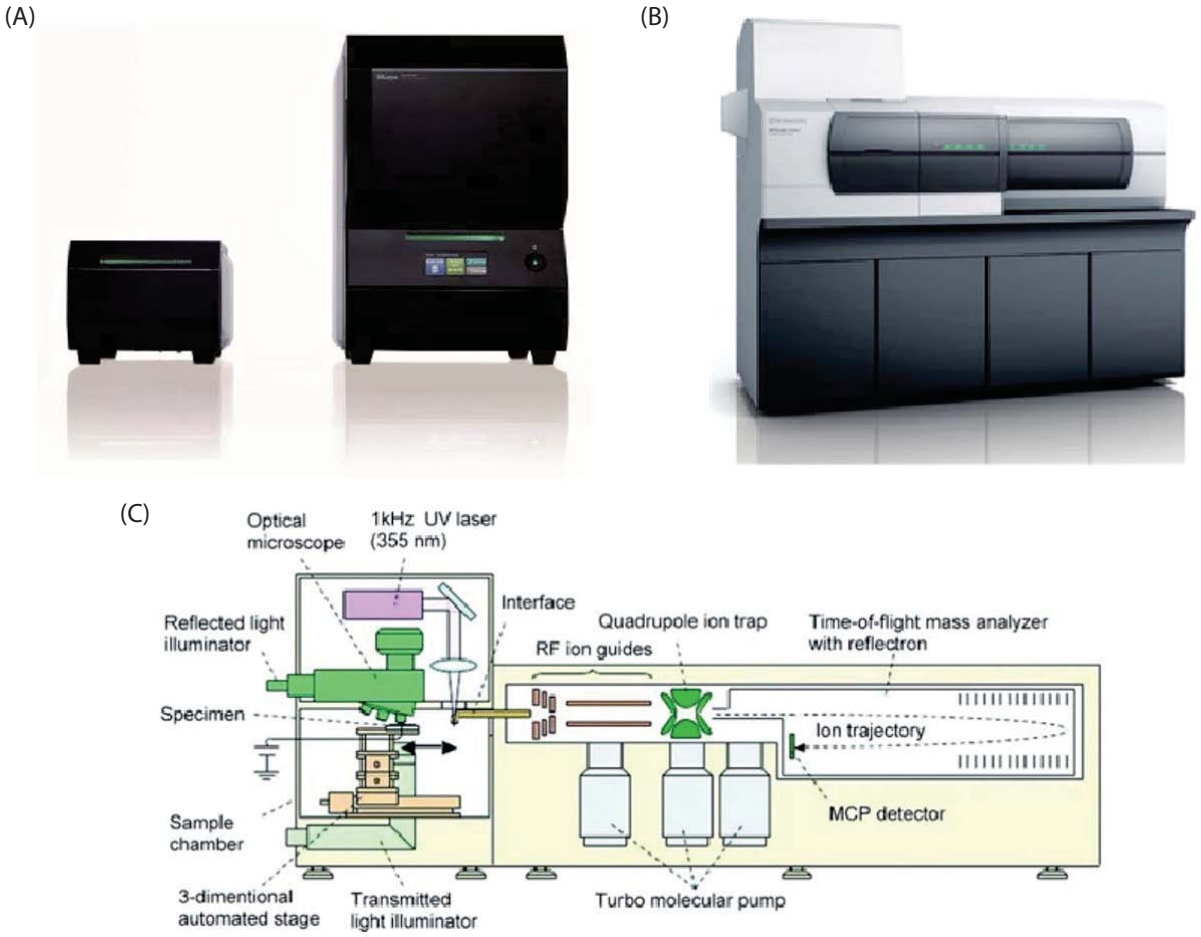


Fig. 2 **Analysis Equipment Used in this Study**

(A) iMLayer ™ used for matrix deposition, (B) iMScope *TRIO* ™ used for measurement, and (C) schematic diagram of the iMScope *TRIO* ™ system. This system is capable of microscopic observation under atmospheric pressure along with ionization using MALDI, and the generated ions are drawn into an ion trap under a vacuum and detected by a time-of-flight mass spectrometer.

4. Experimental Method

In this study, an iMLayer ™ matrix deposition system was used to apply the matrix for MALDI (Fig. 2 (A)). The matrices used were α-cyano-4-hydroxycinnamic acid (α-CHCA, Merck) and 9-aminoacridine (9-AA, Tokyo Chemical Industry Co., Ltd.). These were used for positive mode analysis, and negative mode analysis respectively, and were deposited to a thickness of 0.5 µm on the sample surface by iMLayer. In the positive mode analysis, an additional spray of α-CHCA solution (preparation of 10 mg/ml using 30 % acetonitrile/0.1 % formic acid) was manually performed using an airbrush after the layer deposition [10]. In the positive ion analysis, after 9-AA deposition, 5 % methanol vapor was supplied to the sample surface for 3 seconds for recrystallization [11].

iMScope *TRIO* was used as the measuring system (Fig. 2 (B), (C)). As described above, the iMScope *TRIO* incorporates an optical microscope to capture the surface of the sample under atmospheric pressure, and atmospheric pressure MALDI. The laser for MALDI is an Nd:YAG laser with a repetition frequency of 1 kHz. Ions generated under atmospheric pressure are introduced into the mass analyzer unit via a differential pumping system, and are detected by the ion trap time-of-flight mass spectrometer. The measurable mass range is *m/z* 50 to 3,000, but since the target on this occasion - dexamethasone phosphate ester - is a small-molecule drug, the measurement mass range was set at up to *m/z* 1000.

Fig. 3 (A) shows the flow of measurement for this study. The basic flow is the collection of hair, removal of the cuticle, application of matrix, measurement and imaging with iMScope *TRIO*. The surface of each strand of the collected equine hair was wiped with a rag moistened with distilled water. This process is considered feasible only for MSI, where the sample can be analyzed directly without extraction. Conversely, with the existing method, contamination occurs during extraction if the washing is insufficient. After this process, the hair was dried immediately. The dried hair was affixed to ITO glass slide (Matsunami Glass Ind.,Ltd.) on conductive double-sided tape, and the cuticle was removed from the follicle end of the hair using a microtome blade under a stereomicroscope as shown in Fig. 3 (B). Since the diameter of equine hair is about twice that of human hair - around 200 µm - the surface can be removed easily even in a manual operation. After removal of the cuticle, the hair remaining on the ITO glass slide was taken as the analysis sample, matrix was supplied and it was analyzed.

The drug targeted for the detection in this study was the dexamethasone sodium phosphate (DexaSP) that is a steroidal anti-inflammatory drug. DexaSP could be detected directly in the negative mode using 9-AA matrix. Alternatively, it could also be detected by further pretreating the sample with Girard T reagent (GirT) to derivatize DexaSP for efficient ionization in the positive mode (Fig. 4) [12].

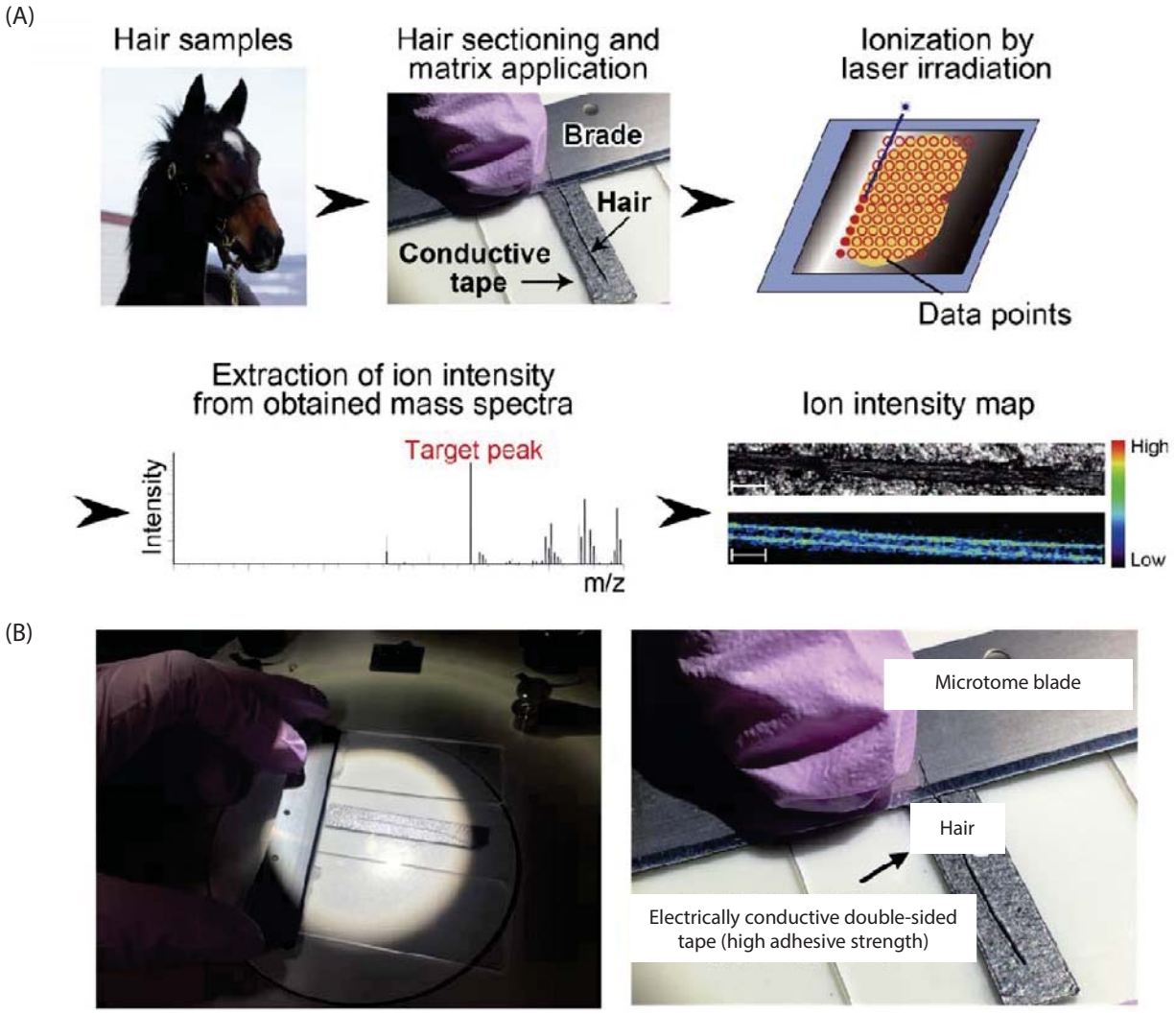


Fig. 3 **(A) Flow of Measurement and (B) Equine Hair Cuticle Removal Method**

The cuticle was removed by using a cryomicrotome blade under a stereomicroscope to expose the hair medulla.

Positive ion detection: GirT derivatized DexaSP

Negative ion detection: Unchanged DexaSP

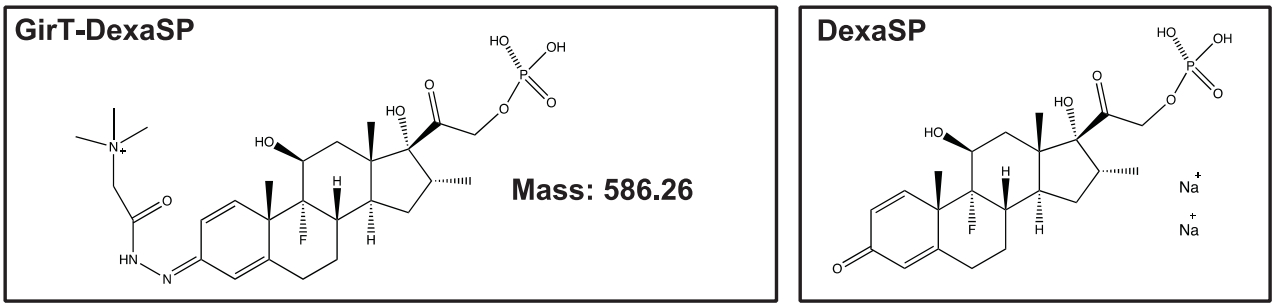


Fig. 4 **Dexamethasone Sodium Phosphate (DexaSP) that is the Target Drug**

For the detection of positive ions, DexaSP derivatives generated with a Girard T reagent were taken as the detection target, and for the detection of negative ions, unchanged DexaSP was taken as the detection target.

5. Result

Fig. 5 shows the results for detection of the positive ions and negative ions using a standard product.

As already mentioned, in positive ion detection GirT derivatized DexaSP was targeted and in negative ion detection unchanged DexaSP was targeted. In positive ion detection, *m/z* 586.267 was obtained as GirT derivatized DexaSP using α-CHCA. This is the peak corresponding to $[GirT\text{-}DexaSP\text{-}2Na+2H]^+$. In negative ion detection on the other hand, a peak was detected at *m/z* 471.160 using 9-AA. This was the peak corresponding to $[DexaSP\text{-}H]^-$.

Peaks derived from DexaSP were observed in both polarities, but because of the time required for pretreatment, and also the fact that the ion intensity is about 100 times higher with the negative ions, it was decided to perform negative ion detection using 9-AA for this equine hair analysis.

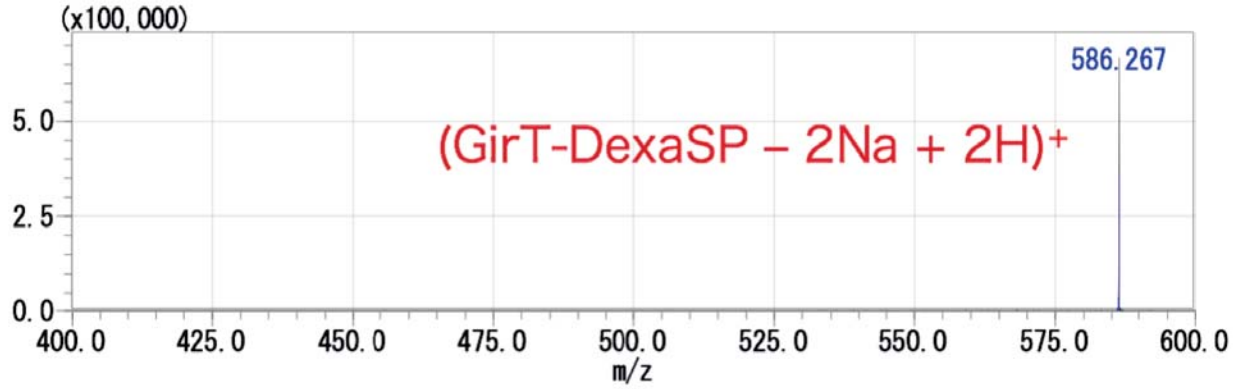
When analyzing the equine hair in question, a control experiment was performed using equine hair from a horse that had not been administered DexaSP and it was confirmed that no *m/z* 471.160 peak was detected (Fig. 6 (A)). Fig. 6 (B) shows the equine hair imaging results from a horse that had been administered dexamethasone phosphate ester.

The sample for this test was equine hair collected on July 13 from a horse that had been injected with 15 to 20 mL of 0.1 % aqueous dexamethasone sodium phosphate (Fujita Pharmaceutical Co., Ltd.) for 3 days in early June 2017. The measurement interval on the iMScope *TRIO* was 80 µm in the x direction and 5 µm in the y direction, and the laser spot size was 2 (system parameters).

In this experiment, 4-cm lengths of equine hair were measured, and they were divided into zones of 1 cm each for that measurement. In Fig. 6 (B) the data is divided into four parts, but the samples were not divided: 4-cm lengths of equine hair were affixed onto ITO glass slides.

Scanning was performed from the follicle toward the tip, and at around 16.48 mm from the follicle a peak derived from unchanged dexamethasone phosphate ester was detected with high intensity. This result is the first case where phosphate esters that are rapidly metabolized in the body have been directly detected from hair and is thought to have made a great impact. Note that peak intensity expressed as absolute intensity is used for the imaging results shown here, and they are presented in multi-color in the intensity range from 300 to 1500. In this data, warm colors mean a high peak intensity.

(A) Positive ion detection: GirT derivatized DexaSP



(B) Negative ion detection: Unchanged DexaSP

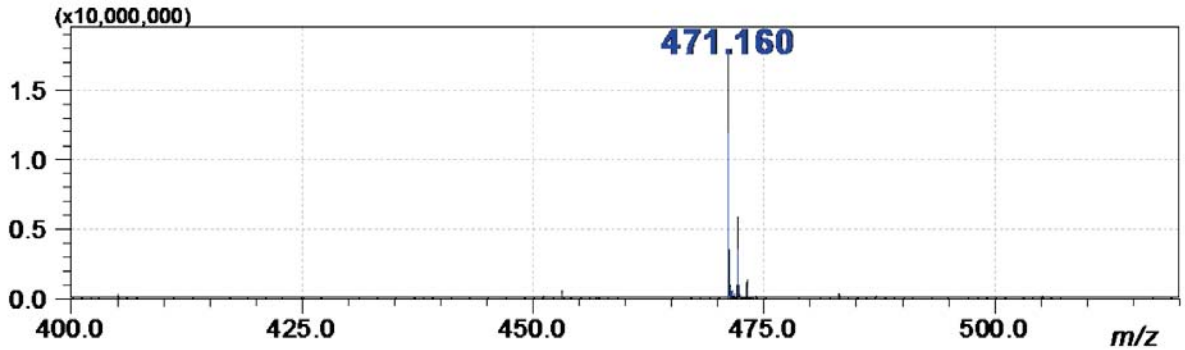


Fig. 5 **Examination of Measurement Conditions Using Standard Products**

It was possible to detect the targeted peak with both positive ion detection and negative ion detection, but negative ion detection was selected due to the simplicity of the pretreatment and the magnitude of the ion intensity.

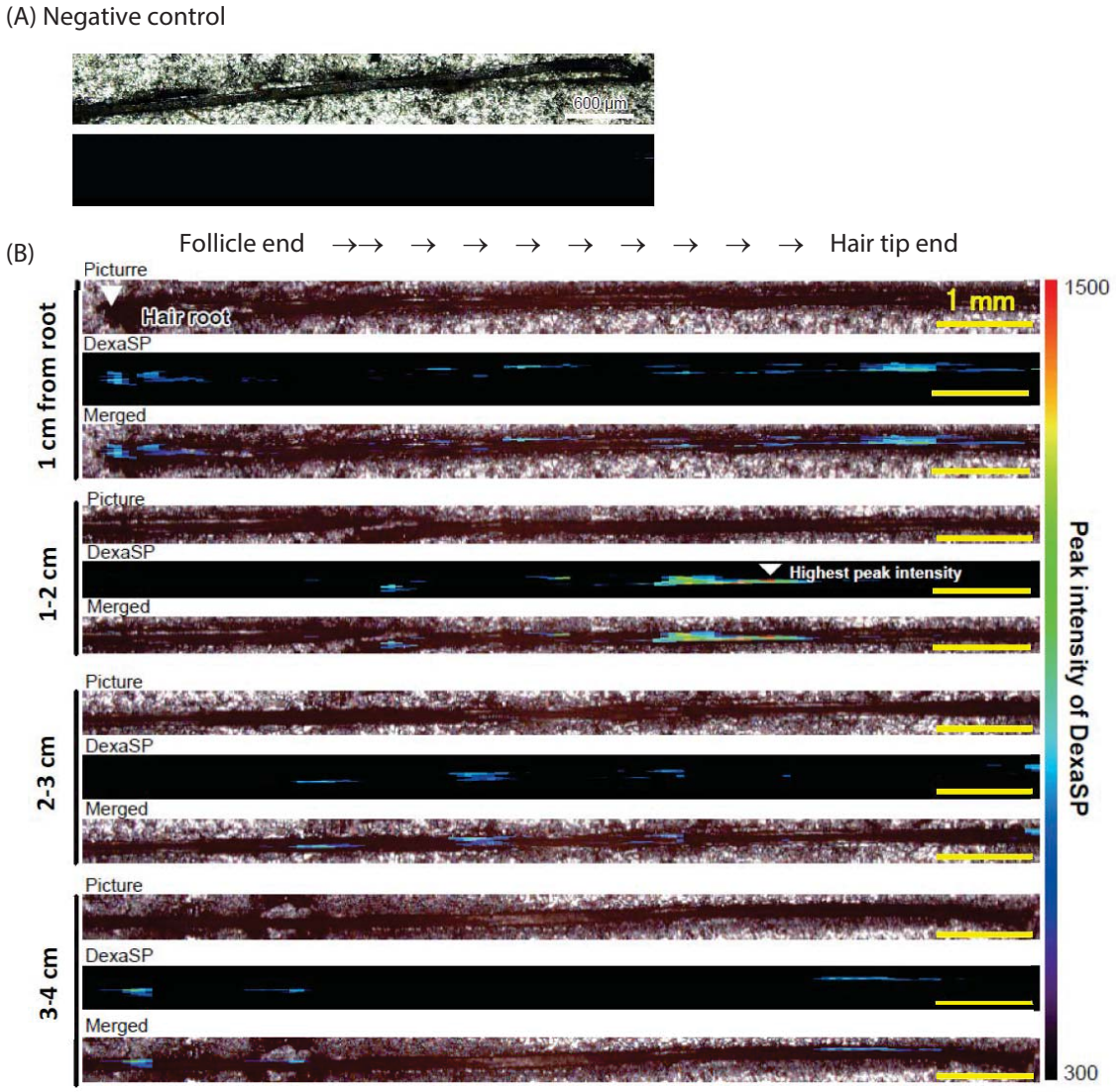


Fig. 6 **Measurement of DexaSP Distribution in Equine Hair**

(A), the distribution in equine hair from a horse that had not been administered DexaSP, which was the negative control, and (B), the distribution in equine hair from a horse that had been administered DexaSP (15 to 20 mL of 0.1% aqueous dexamethasone sodium phosphate injection from Fujita Pharmaceutical Co. was administered for 3 days at 1.315 mg/mL) were measured. A length of 4 cm from the follicle was scanned with an iMScope *TRIO* ™. The results at 1 cm intervals were recorded. The maximum peak intensity range was observed in the region of 16.48 mm from the hair follicle. Since equine hair grows at a rate of 2.0 cm per month on average, it can be determined that the drug was administered 25 days prior to the sampling date.

6. Discussion

In this experiment, we consider that we managed to detect the targeted drug in equine hair, in view of the measurement results for the negative control chosen based on the ionization conditions. In equine hair from a horse that had been administered the drug, localization was observed at the 16.48 mm position. The growth rate of equine hair is known to be 2 cm per month on average, which is twice that of human beings. When the time of administration was estimated based on the distance from the hair follicle shown by this rate of growth and the maximum intensity, it was found to be around 24 to 25 days after administration. The administration record showed that the drug had been administered approximately one month prior to collection of the hair, and the localization is considered correct based on comparison with that information.

On the other hand, it can be seen that some signal is detected in the vicinity of the hair follicle although the ion intensity is low. On checking the mass spectrum this was found to be a signal derived from noise, and further improvements in ionization efficiency and signal to noise ratio are considered essential to deal with real samples. In order to achieve this, there is a possibility that the matrix application method may have to be devised or another matrix may need to be selected.

7. Summary and Future Prospects

Dexamethasone sodium phosphate is an anti-inflammatory drug whose use is permitted, but its use before a race is prohibited [13], and in recent years its detection after the race in the Tokyo Grand prize winning horse Apollo Kentucky in December 2016 is still fresh in the memory. The results of this experiment can be said to be the first example of the possibility of applying the combination of matrix application by iMLayer and measurement by iMScope *TRIO* to doping tests.

Further, since phosphate esters are rapidly metabolized in the body, the point that the unchanged drug was detected directly in the hair is also considered very significant. On the other hand, since there is a lot of noise in the imaging results, it is considered necessary to make further improvements to the hair pretreatment in order to improve ion intensity. Another element that is considered essential from here on is study of the quantitative performance and of the drugs that can be detected (including anabolic steroids). So although there are many problems still to be resolved for this application, we consider that we were able to show the potential of iMScope *TRIO*.

8. Possibilities of Equine Hair Analysis

Currently, there is a lot of debate about doping control for racehorses around the world, and an international conference to discuss racehorse doping tests and protection against racehorse injuries (ICRAV: International Conference of Racing Analysts and Veterinarians) is held once every two years. In 2018, it was held in Dubai in the United Arab Emirates and the author participated for the first time and presented the results of this study. Fig. 7 shows the conference hall and a view of the Meydan Racecourse. Being able to present this research in the conference hall next to the Meydan Racecourse, one of the world's premier racetracks, has been one of the most memorable events of my life so far.

Usually, the participants from Japan at this conference are people associated with the JRA or researchers from the Laboratory of Racing Chemistry, and the only participant from a university was the author. However, researchers from the Hong Kong Jockey Club, the Australian Turf Club and elsewhere have a great deal of interest and high expectations about drug detection using IMS, and the discussion was very lively. In November 2018 while writing this article, Boldenone, which is a banned drug, was detected in the horse Ubatouban that was running in the Iwate horse races [14]. In the coming future I would like to continue to establish the technology, including simplification of the hair pretreatment technique, so that this new measurement method from Japan can be used for the detection of doping in the world's racehorses.

I also received a lot of cooperation in advancing this research not just from Shimadzu Corporation but also from the entire staff of Equine Racing Co., Ltd., whose representative is the co-author.
I will finish this article by posting pictures of equine hair sampling, along with a recent photograph of the author and co-author, in Fig. 8.

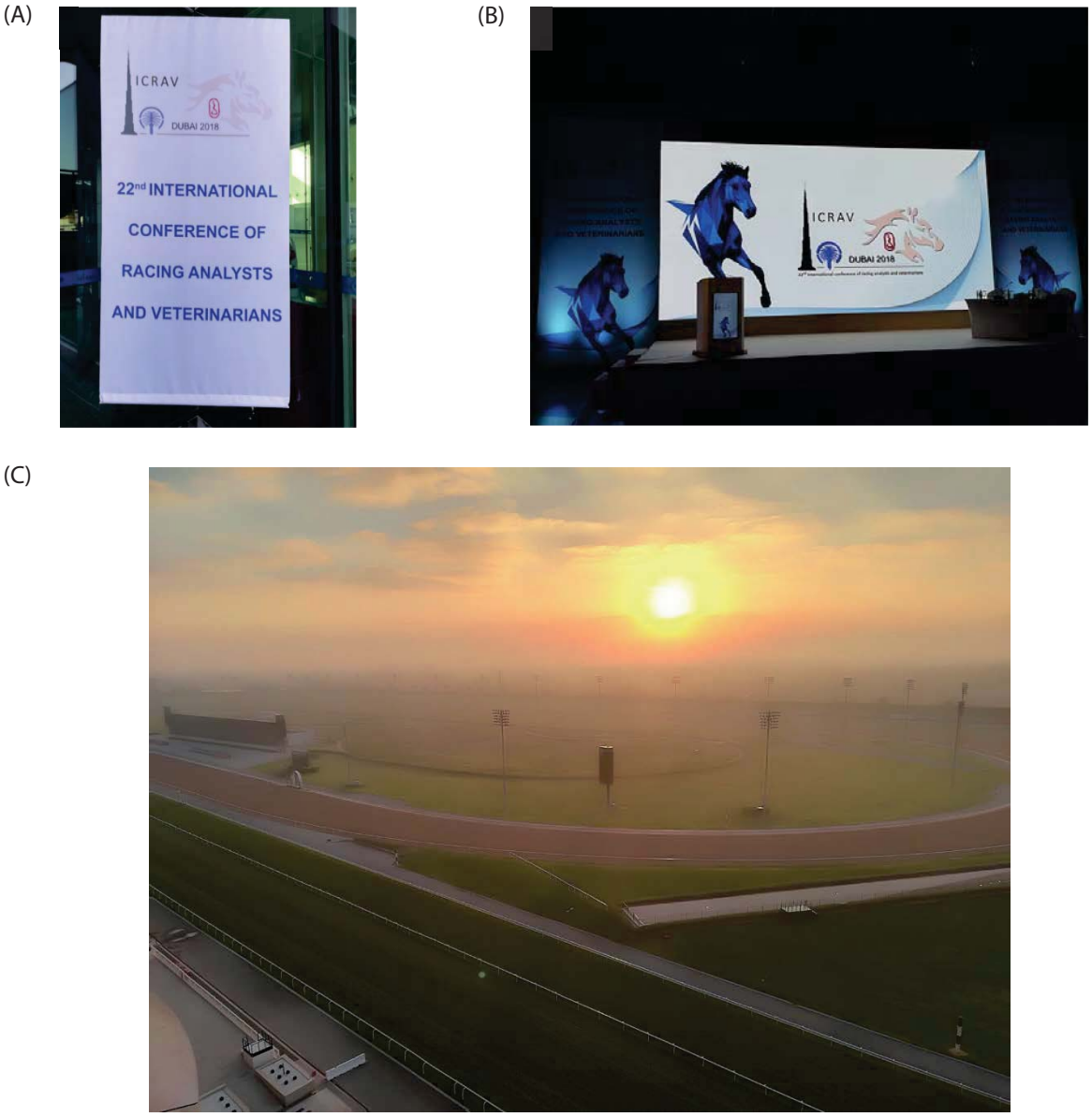


Fig. 7 **ICRAV2018**

(A), (B) Scenes at the ICRAV 2018 venue, and (C) morning at the Meydan racecourse, where ICRAV was held.
The Meydan racecourse was a spectacular sight, with a scale and opulence unparalleled in Japan.