Fujii I[*1], Hashimoto M[*1], Konishi K[*1], Unezawa A[*1], Sakuraba H[*1], Suzuki K[*1], Tsushima H[*1], Iwasaki M[*1], Yoshida S[*1], Kudo A[*1], Fujita R[*1], Hichiwa A[*1], Saito K[*1], Asano T[*1], Ishikawa J[*2], Wakana D, Goda Y, Watanabe A[*3], Watanabe M[*3], Masumoto Y[*3], Kanazawa J[*3], Sato H[*3], Uchiyama M[*3]: Shimalactone Biosynthesis Involves Spontaneous Double Bicyclo-Ring Formation with 8π-6π Electrocyclization.

Angew Chem Int Ed. 2020;59:8464-70. doi: 10.1002/anie.202001024

Shimalactones A and B are neuritogenic polyketides possessing characteristic oxabicyclo[2.2.1]heptane and bicyclo[4.2.0]octadiene ring systems that are produced by the marine fungus *Emericella variecolor* GF10. We identified a candidate biosynthetic gene cluster and conducted heterologous expression analysis. Expression of ShmA polyketide synthase in *Aspergillus oryzae* resulted in the production of preshimalactone. *Aspergillus oryzae* and *Saccharomyces cerevisiae* transformants expressing ShmA and ShmB produced shimalactones A and B, thus suggesting that the double bicyclo-ring formation reactions proceed non-enzymatically from preshimalactone epoxide. DFT calculations strongly support the idea that oxabicyclo-ring formation and 8π-6π electrocyclization proceed spontaneously after opening of the preshimalactone epoxide ring through protonation. We confirmed the formation of preshimalactone epoxide in vitro, followed by its non-enzymatic conversion to shimalactones in the dark.

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Izutsu K, Usui A, Yamamoto E, Abe Y, Yoshida H, Goda Y: Effect of complex coacervation with hyaluronic acid on protein transition in a subcutaneous injection site model system. *Chem. Pharm. Bull*. 2020;68:1109-12. doi: 10.1248/cpb.c20-00585

The occurrence of complex coacervation in an aqueous mixture of proteins (lysozyme, albumin, immunoglobulin G) and hyaluronic acid and its effect on protein transition in a model system was studied to elucidate factors determining the bioavailability of subcutaneously injected therapeutic proteins. Mixing of hyaluronic acid and the model proteins induced complex coacervation at solution pH close to or below the isoelectric point of the proteins. In vitro dialysis using membranes with large pore size tube represented a limitation in the protein transition of the coacervation mixture. Thermal analysis suggested there was retention of the protein conformation in the polymer complex.

Keywords: coacervation, subcutaneous, hyaluronic acid

吉田寛幸，阿部康弘，臼井明子，伊豆津健一：シクレソニド吸入剤の肺炎患者による使用を想定したスペーサーの影響検討.

薬学雑誌，2020;140(12):1495-500. doi: 10.1248/yakushi.20-00169

Achieving appropriate inhalation in patients with coronavirus disease 2019 (COVID-19) is a common challenge in the use of repurposed metered-dose inhaler (MDI) formulations. The purpose of this study was to evaluate the effect of five valved holding chambers (VHCs) on the inhalation of ciclesonide from Alvesco MDI. The aerodynamic particle size distribution of ciclesonide discharged from Alvesco MDI was evaluated using a Next Generation Impactor in the presence and absence of VHCs. The use of VHCs retained or slightly increased the amount of ciclesonide in the fine particle diameter range (aerodynamic particle size below 3 μm) (FPD) and reduced the amount at the induction port after coordinated inhalation. However, the use of VHC reduced the FPD of the formulation by increasing the time between the MDI discharge and the pump suction by various degrees among the five VHCs. These results indicated that use of the VHCs and minimizing the inhalation delay time should ensure sufficient inhalation of ciclesonide particles.

Keywords: valved holding chamber, metered-dose inhaler, fine particle dose

Yoshida H, Usui A, Abe Y, Goda Y, Izutsu K: Relationship between geometric and aerodynamic particle size distribution in the formulation of solution and suspension metered-dose inhalers.

AAPS PharmSciTech. 2020;21(5):158. doi: 10.1208/s12249-020-01675-3

The relationship between the geometric particle size distribution (GPSD) and the aerodynamic particle size distribution (APSD) of commercial solution and suspension metered-dose inhaler (MDI) formulations was assessed to clarify the use of GPSD to estimate the APSD. The size distribution of particles discharged from four suspension and four solution MDIs was measured using the Inas®100 light-scattering spectrometer and a Next Generation Impactor. The conversion factor was calculated by measuring the GPSD and APSD of MDIs. The morphology and physical properties of MDIs were studied using scanning electron microscopy (SEM) and differential scanning calorimetry (DSC). Six of the eight MDIs showed similar conversion factor profiles, irrespective of their composition and formulation types. Applying the conversion factor obtained from one of the six MDIs resulted in a particle size distribution comparable to each APSD except for some formulations. The two other solution MDIs, which contained citric acid, had much higher and variable conversion factors. SEM images and DSC scans of the solids obtained by nebulization of the solutions containing beclomethasone and/or citric acid showed the formation of a paste-like amorphous solid. These results indicated that APSD of solution and suspension MDIs that form rigid particles may be estimated by using the conversion factor and GPSD. Contrarily, the estimation is more difficult in formulations that tend to lose the particle structure during the measurement.

Keywords: optical particle size, aerodynamic particle size, conversion factor

Yoshida H, Abe Y, Tomita N, Izutsu KI. Utilization of Diluted Compendial Media as Dissolution Test Solutions with Low Buffer Capacity for the Investigation of Dissolution Rate of Highly Soluble Immediate Release Drug Products.

Chem Pharm Bull. 2020;68(7):664-70. doi: 10.1248/cpb.c20-00247

Research from the past decade has shown that the buffer capacities of intestinal fluids are much lower than those in the media used for dissolution test of many solid formulations. The purpose of this study was to elucidate the effect of buffer capacity on the dissolution profiles of highly soluble drug products, using metoclopramide (a biopharmaceutics classification system [BCS] class III drug) tablets as a model. The dissolution profiles of three metoclopramide products were obtained in Japanese pharmacopeia dissolution medium (pH 1.2 and 6.8), diluted medium with low buffer capacity comparable to that of gastrointestinal fluid, and other biorelevant media. One product showed slower dissolution in the medium with lower buffer capacity (bio-relevant, diluted compendial solution), but substantially similar dissolution in the compendial test solutions. Disintegration difference was implied to be involved in the different dissolution profiles depending on the medium buffer capacity. This study indicated the importance of media buffer capacity as a factor inducing different dissolution between products of highly soluble active pharmaceutical ingredients. The diluted compendial media would be a useful alternative to biorelevant media for the detection of the different formulation performances depending on the buffer capacities.

Keywords: buffer capacity, dissolution test, highly soluble drug

Abe Y, Yamamoto E, Yoshida H, Usui A, Tomita N, Kanno H, Masada S, Yokoo H, Tsuji G, Uchiyama N, Hakamatsuka T, Demizu Y, Izutsu KI, Goda Y, Okuda H: Temperature-Dependent Formation of *N*-Nitrosodimethylamine during the Storage of Ranitidine Reagent Powders and Tablets.

Chem Pharm Bull. 2020;68(10):1008-12. doi: 10.1248/cpb.c20-00431

The purpose of this study was to elucidate the effect of high-temperature storage on the stability of ranitidine, specifically with respect to the potential formation of *N*-nitrosodimethylamine (NDMA), which is classified as a probable human carcinogen. Commercially available ranitidine reagent powders and formulations were stored under various conditions, and subjected to LC-MS/MS analysis. When ranitidine tablets from two different brands (designated as tablet A and tablet B) were stored under accelerated condition (40℃ with 75% relative humidity), following the drug stability guidelines issued by the International Conference on Harmonisation (ICH-Q1A), for up to 8 weeks, the amount of NDMA in them substantially increased from 0.19 to 116 ppm

and from 2.89 to 18 ppm, respectively. The formation of NDMA that exceeded the acceptable daily intake limit (0.32 ppm) at the temperature used under accelerated storage conditions clearly highlights the risk of NDMA formation in ranitidine formulations when extrapolated to storage under ambient conditions. A forced-degradation study under the stress condition (60℃ for 1 week) strongly suggested that environmental factors such as moisture and oxygen are involved in the formation of NDMA in ranitidine formulations. Storage of ranitidine tablets and reagent powders at the high temperatures also increased the amount of nitrite, which is considered one of the factors influencing NDMA formation. These data indicate the necessity of controlling/monitoring stability-related factors, in addition to controlling impurities during the manufacturing process, in order to mitigate nitrosamine-related health risks of certain pharmaceuticals.

Keywords: *N*-nitrosodimethylamine, forced degradation, ranitidine

Miyazaki T, Kanno H, Yamamoto E, Ando D, Izutsu K, Goda Y: Cold Flow Evaluation in Transdermal Drug Delivery Systems by Measuring the Width of the Oozed Adhesive.

AAPS PharmSciTech. 2020;21:120. doi: 10.1208/s12249-020-01661-9

The objective of this study was to develop a simpler and more practical quantitative evaluation method of cold flow (CF) in transdermal drug delivery systems (TDDSs). CF was forcibly induced by loading a weight on a punched-out sample (bisoprolol and tulobuterol tapes). When the extent of CF was analyzed using the area of oozed adhesive as following a previously reported method, the CF profiles were looked different between the samples 12 mm in diameter subjected to a 0.5-kg weight and samples 24 mm in diameter subjected to a 2.0-kg weight despite an equal load per unit area (4.42 g/mm^2). The width of oozed adhesive around the original sample was suggested to be an index that properly describes the relationship between the load per unit area and the extent of CF. Further, it was clarified that the average CF width over the entire circumference of the sample was the same whether the samples were round or square as long as the sample area and load were the same. We also observed a linear relationship between the CF width and the aspect ratio of oval and rectangular samples. These results indicated that the CF properties of typical TDDS products lacking CF-proof processing at the edges could be determined by testing samples cut from the product rather than the whole TDDS patch. The proposed width measuring method was simple and useful for optimizing the composition of the adhesive and for testing the quality of the product.

Keywords: cold flow, transdermal drug delivery system, adhesive

Kato M*, Athumi Y*, Yamaguchi M*, Date H*, Yamamoto E, Murayama S*, Karasawa K*: Trimethylammonium modification of a polymer-coated monolith column for rapid and simultaneous analysis of nanomedicines.

J Chromatogr A. 2020;1617(26):460826. doi: 10.1016/j.chroma.2019.460826

Drug-containing nanoparticles (nanomedicine) are ideal targeted-drug-delivery systems. However, methods for the simultaneous analysis of the drug within the nanoparticle and free drug in a short time are rather limited. In this study, we developed a polymer-modified monolithic column with cationic groups (trimethylammonium) for the simultaneous analysis of the drug within the nanoparticle and the free drug. The use of the acrylamide group was determined as the optimum connecting group, and the optimum concentration of the modifier was 6%. The prepared column retained the drug within the nanoparticle by anion exchange, and its elution time was controlled by the ionic concentration (tris(hydroxymethyl)aminomethane, Tris) of the mobile phase. The separation of two typical nanomedicines was studied on the prepared column. For DOXIL and Abraxane, the drugs within the nanoparticle were well separated from the free drugs, on the developed column. The developed polymer-coated monolithic column with trimethylammonium modification is expected to enable the rapid analysis of various nanomedicines.

Keywords: nanomedicine, drug, anion exchange

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Yamamoto E, Taquahashi Y, Kuwagata M, Saito H, Matsushita K, Toyoda T, Sato F*, Kitajima S, Ogawa K, Izutsu K, Saito Y, Hirabayashi Y, Iimura Y, Honma M, Okuda H, Goda Y: Visualizing the spatial localization of ciclesonide and its metabolites in rat lungs after inhalation of 1-μm aerosol of ciclesonide by desorption electrospray ionization-time of flight mass spectrometry imaging.

Int J Pharm. 2021;595:120241. doi: 10.1016/j.ijpharm.2021.120241

Inhaled ciclesonide (CIC), a corticosteroid used to treat asthma that is also being investigated for the treatment of corona virus disease 2019, hydrolyzes to desisobutyryl-ciclesonide (des-CIC) followed by reversible esterification when exposed to fatty acids in lungs. While previous studies have described the distribution and metabolism of the compounds after inhalation, spatial localization in the lungs remains unclear. We visualized two-dimensional spatial localization of CIC and its metabolites in rat lungs after administration of a single dose of a CIC aerosol (with the mass median aerodynamic diameter of 0.918-1.168 μm) using desorption electrospray ionization-time of flight mass spectrometry imaging (DESI-MSI). In the analysis, CIC, des-CIC, and des-CIC-oleate were imaged in frozen lung sections at high spatial and mass resolutions in negative-ion mode. MSI revealed the coexistence of CIC, des-CIC, and des-CIC-oleate on the airway epithelium, and the distribution of des-CIC and des-CIC-oleate in peripheral lung regions. In addition, a part of CIC independently localized on the airway epithelium. These results suggest that distribution of CIC and its metabolites in lungs is related to both the intended delivery of aerosols to pulmonary alveoli and peripheral regions, and the potential deposition of CIC particles on the airway epithelium.

Keywords: mass spectrometry imaging, aerodynamic diameter, inhalation

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Ozawa Y*, Watanabe Y*, Ando D, Koide T, Fukami T*: Advanced Formulation Design for Topical Creams Assisted with Vibrational Spectroscopic Imaging.

Chem Pharm Bull. 2021;69(3):271-7. doi: 10.1248/cpb.c20-00979.

Vibrational spectroscopic imaging has become useful analytical tools for quality control of drug products. In this study, we applied microscopic attenuated total reflection (ATR)-IR and confocal Raman microscopy to elucidate microscopic structure of creams and for the formulation design in the development of semi-solid drug products. The model creams were prepared with prednisolone (PRD) and fluconazole (FLC) as active pharmaceutical ingredients and oily solvents such as mineral oil (MO), isopropyl myristate (IPM), benzyl alcohol (BA) and diethyl sebacate (DES). As a result of microscopic ATR-IR imaging, several domains indicating oily internal phase were observed, which had absorption around 1732 and 1734 cm^{-1} derived from MO, IPM and DES. In addition, domains of BA around 1009 cm^{-1} were observed at the complemental or similar position in the formulation with MO or DES, respectively. These results suggested that the creams were oil-in-water type and the distribution of domains would reflect the compatibility of the solvents. The contents of PRD and BA were determined quantitatively in each layer after the intentional separation of the creams and the results agreed well with the imaging analysis. Whereas, confocal Raman imaging allowed to visualize the distribution of the components in depth direction as well as two-dimensional plane. In particular, the Raman imaging would ensure the coexistence of FLC and BA as oily phase in the cream. From these results, the feasibility of spectroscopic imaging techniques was successfully demonstrated for the formulation design of semi-solid dosage forms.

Keyword: confocal Raman spectroscopy, formulation study, imaging analysis

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Takaku T*1, Hattori Y*1, Sasaki T*2, Sakamoto T, Otsuka M*1: Evaluation of swelling properties and drug release from mechanochemical pre-gelatinized glutinous rice starch matrix tablets by near infrared spectroscopy.

J Near-Infrared Spec. 2021;29:92-101. doi: 10.1177/0967033520982351

The effect of grinding on the pharmaceutical properties of matrix tablets consisting of ground

glutinous rice starch (GRS) and theophylline (TH) was predicted by near infrared (NIR) spectroscopy. Ground GRS samples were prepared by grinding GRS in a planetary ball mill for 0-120 min, measured by X-ray diffractometry (XRD) and NIR, and then evaluated for crystallinity (%XRD) based on XRD profiles. Tablets containing TH (5 w/w%), ground GRS (94 w/w%), and magnesium stearate (1 w/w%) were formed by compression. Gel-forming and drug-release processes of the tablets were measured using a dissolution instrument with X-ray computed tomography (XCT). Swelling ratio (SWE) and mean drug-release time (MDT) were evaluated based on XCT and drug-release profiles, respectively. Calibration models for predicting percent %XRD, MDT, and SWE were constructed based on the NIR of ground GRS using partial least-squares. The results indicated the possibility of controlling the pharmaceutical properties of matrix tablets by altering the pre-gelatinization of GRS based on changes in their NIR spectra during the milling process.

Keywords: NIR, swelling properties drug release

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Omata R*1, Hattori Y*1, Sasaki T*2, Sakamoto T, Otsuka M*1: Elucidation of the molecular mechanism of wet granulation for pharmaceutical standard formulations in a high-speed shear mixer using near infrared spectroscopy.

Pharmaceuticals. 2020;13: 226. doi: 10.3390/ph13090226

The granulation process of pharmaceutical standard formulation in a high-speed shear wet granulation (HSWG) was measured by in-line near-infrared spectroscopy (NIRS) and agitation power consumption (APC) methods. The F-1, F-2, and F-3 formulations (500 g) contained 96% *w/w* α-lactose monohydrate (LA), potato starch (PS), and a LA:PS = 7:3 mixture, respectively, and all the formulations contained 4% *w/w* hydroxypropyl cellulose. While adding purified water at 10 mL/min, the sample powder was mixed. The calibration models to measure the amount of binding water (Wa) and APC of the HSWG formulations were established based on NIRS of the samples measured for 60 min by partial least-squares regression analysis (PLS). Molecular interaction related to APC between the particle surface and binding liquor was analyzed based on NIRS. The predicted values of Wa and APC for all formulations were superimposed with the measured values on a straight line, respectively. The regression vector (RV) of the calibration model for Wa indicated the chemical information of all the water in the samples. In contrast, the RV for APC suggested that APC changes in the processes are related to powder aggregation because of surface tension of binding water between particles.

Keywords: high-speed shear wet granulation, agitation powder consumption, monitoring by in-line near-infrared spectroscopy

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Koyanagi K*1, Ueno A*1, Hattori Y*1, Sasaki T*2, Sakamoto T, Otsuka M*1: Analysis of granulation mechanism in a high-shear wet granulation method using near-infrared spectroscopy and stirring power consumption.

Colloid Polymer Sci. 2020;298:977-87. doi: 10.1007/s00396-020-04655-y

The dynamic granulation process of high-speed shear wet granulation (HSWG) was measured by in-line near-infrared spectroscopy (NIRS) and agitation power consumption (APC) methods. Molecular interactions between powder particles and the binding liquid were analyzed based on both NIRS and APC data by multivariable regression analysis. The granulated sample used glass beads (d_{50} = 46 μm) with or without hydroxypropyl cellulose, and the binder solution used purified water. The HSWG granulator (2-L volume) with APC device and NIRS was used, and the agitator was rotated at 600 min^{-1} and the chopper at 2000 min^{-1} with glass beads to be granulated being 920 g (0.6 L), and a total of 360 mL of purified water was added at 10 mL/min. In order to establish calibration models to predict APC and amount of binding water of the granular formulations, NIRS spectra of the granular samples were recorded every 10 s for 40 min. The calibration models to predict moisture content and APC were constructed based on the corrected NIRS spectral data by partial least-squares regression (PLSR) analysis. The relationships between actual and predicted values for moisture

content and APC produced a straight line, respectively. The regression vector (RV) of the PLS model to predict the water content showed the presence of free water between the bead powder particles. On the other hand, the RV for the APC showed the presence of bound water between the particles.

Keywords: high-speed shear wet granulation, in-line near-infrared spectroscopy, process analytical technology

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Sasaki T*1, Sakamoto T, Otsuka M*2: Precise Evaluation of the Effects of a Small Amount of D-histidine in L-histidine Crystal Form B Using High-Frequency-Accurate Terahertz Spectroscopy.
J Infrared Millimeter Terahertz Waves. 2020;41:529-41. doi: 10.1007/s10762-020-00675-5

The effects of small amounts of enantiomeric impurities on the vibrational modes of polycrystalline L-histidine (His) were examined using high-frequency-accurate terahertz (THz) spectroscopy. Samples of polycrystalline L-His in its monoclinic form (form B) were grown from an ethanol-rich solvent. D-histidine, ranging from 0.05 to 10.0% in content, was added to the L-His/ethanol-rich solution prior to recrystallization. The crystal forms of all recrystallized samples were first confirmed by high-frequency-accurate THz spectra recorded at 70 K and room-temperature powder X-ray diffraction spectra. A detailed analysis of two spectral features (1.54 and 1.80 THz) measured at 10 K showed that the absorption lines had a narrow linewidth, as well as broad spectral components that could be fitted by Gaussian curves corresponding to defects within the crystals. The shifts in peak frequency with varying amounts of D-His additive in the polycrystalline L-His samples were examined in detail. It was observed that the peak frequency shifted to lower frequencies when small amounts of D-histidine were present and then to higher frequencies when relatively large amounts were present. At low concentrations, D-His molecules are thought to act as impurities that disturb the normal mode vibrations of L-His crystals; however, above certain concentrations, D-His contributes to the formation of a DL racemic crystal. Although the effects of small amounts of D-His molecules in L-His crystals were detectable, quantitative determination of trace amounts of impurity was limited by the complex crystallography of L-His, which includes defects and racemic compounds.

Keywords: terahertz spectroscopy, impurity detection, racemic crystal

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Gato K*1, Fujii MY*1, Hisada H*1, Carriere J*2, Koide T, Fukami T*1: Molecular state evaluation of active pharmaceutical ingredients in adhesive patches for transdermal drug delivery.
J Drug Deliv Sci Technol. 2020;58:101800. doi: 10.1016/j.jddst.2020.101800

In this study, we prepared mock patches consisting of model drug (felbinac) and three types of acrylic polymers with different functional substituent and/or physical properties, and evaluated the correlation of pharmaceutical properties and molecular state of felbinac in these patches. Polarizing microscopic observation, powder X-ray diffraction and Raman spectroscopy were employed and then different propensity was observed in the crystallization behaviour and precipitated crystal form in each patches. In particular, Raman spectra in low frequency region were useful to detect polymorphic change in patches. 1 H NMR was also used to investigate the interaction between felbinac and the polymers. In addition, pharmaceutical properties of mock patches were evaluated by dissolution test and in vitro skin permeation test. From these results, crystallization of felbinac was occurred in the case of weaker interaction between felbinac and the polymer, which has carboxy group as substituent, and resulted in higher drug release and skin permeation. In conclusion, it was essential to consider the compatibility of drug and polymer, which constituted adhesive layer in patches, in molecular level.

Keywords: low frequency Raman spectroscopy, transdermal drug delivery system, crystal form

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Inoue M[*1], Hisada H[*1], Takatori K[*1], Koide T, Fukami T[*1], Roy A[*2], Carriere J[*2]: Solid-State Analysis of α-Cyclodextrin Inclusion Complexes Using Low-Frequency Raman Spectroscopy.
Anal Chem 2021;93(2):704-8. doi: 10.1021/acs.analchem.0c03854

A rapid and nondestructive analytical technique is critical for the analysis of cyclodextrin inclusion complexes in solid dosage forms. This study proposed a newly developed low-frequency Raman spectroscopy as a candidate technique for the analysis of cyclodextrin inclusion complexes. In this study, we selected a typical series of five crystalline cyclodextrin inclusion complexes and reported the usefulness of Raman spectroscopy for analyzing these inclusion complexes. Some inclusion complexes clearly differed from the raw materials in conventional Raman spectra. In another case, though specific differences were not observed between inclusion complexes and raw materials in conventional Raman spectra, clear differences were observed in low-frequency Raman spectra. Moreover, no characteristic differences between inclusion complexes consisting of different guest molecules were observed in conventional Raman spectra. The characteristic differences were observed only in low-frequency Raman spectra. Therefore, low-frequency Raman spectroscopy is a useful technique for solid-state analysis of crystalline inclusion complexes.

Keywords: oligosaccharide, Raman spectroscopy, molecular interaction

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Yamamoto Y[*1], Odutsumi A[*1], Miwa E[*1], Fukami T[*2], Koide T: Evaluation of the factors contributing to the stability of the mixture of heparinoid oil-based cream and droplet dispersion-type ointment.
J Drug Deliv Sci Technol. 2021;61:102218 doi: 10.1016/j.jddst.2020.102218

We tried to clarify the pharmaceutical properties of heparinoid oil-based cream (HPOC) formulations from various aspects, such as rheological properties. Furthermore, the stability of the mixture of droplet dispersion-type betamethasone butyrate propionate ointment (BBPO) formulations and all HPOC formulations at various mixing ratios was evaluated. Next, we prepared a model droplet dispersion-type ointment and evaluated the relationship between the surfactant contained in the droplet dispersion-type ointment and the mixture stability. The present study revealed that two HPOC formulations have a lower viscosity than other HPOC formulations. Bleeding occurred in the mixture of the two HPOC formulations and droplet dispersion-type BBPO formulation at a mixing ratio of 1:1, suggesting that the pharmaceutical properties of the two HPOC formulations influence the stability of the mixture. A study using the time-domain-nuclear magnetic resonance (TD-NMR) revealed that the molecular mobility of water in those mixtures was diverse. When the stability of the mixture of the model droplet dispersion-type ointment and all the HPOC formulations was evaluated, it was stable in all mixtures when the HLB value of the surfactant added to the model ointment formulation was low (approximately 5). In contrast, it was unstable in all the mixtures when the HLB value of the surfactants was high (approximately 16). In the case of surfactants, such as polyoxyethylene hydrogenated castor oil 40, contained in droplet dispersion-type BBPO formulations, where the HLB value is middle (approximately 12), the mixture stability depends on the pharmaceutical properties of HPOC formulations. Therefore, bleeding occurred only in mixtures with the two HPOC formulations. As described above, to predict the stability of a mixture, it is necessary to understand the pharmaceutical properties of both components from various aspects.

Keywords: betamethasone butyrate propionate, droplet dispersion-type ointment, stability

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Sakuda M[*1], Yoshida N[*1], Koide T, Kimura K[*1], Tsuboi H[*1]: Clarification of the internal structure and factors of poor dissolution of substandard roxithromycin tablets by near-infrared chemical imaging.
Int J Pharm 2021;596:120232 doi: 10.1016/j.ijpharm.2021.120232

The spread of substandard and falsified medicines

has become a global problem, especially in low- and middle-income countries (LMICs). Previously, we found that some tablets containing the same active ingredient had large differences in their dissolution even though their contents were comparable. In this study, we investigated the poor dissolution of roxithromycin tablets using near-infrared chemical imaging (NIR-CI) to visualize the internal tablet structure. Roxithromycin tablets collected in LMICs and the pioneer product Rulid® as a reference were cut to a flat surface for analysis. NIR spectral data were normalized, and a principal component analysis was performed to create a tablet internal structure image. For Rulid®, the differences between the spectra with high and low scores were small, and well-defined aggregation of ingredients was not observed. However, large differences in the scores were found for roxithromycin tablets manufactured in some LMICs, and non-uniformity of ingredient distribution and aggregation were observed. Additionally, some pharmaceutical excipients, such as starch or magnesium stearate, were found in certain aggregates by comparing NIR spectra. The NIR-CI results showed some excipients existed as large aggregates, which indicated that the ingredients were not evenly mixed in the roxithromycin tablet, and this contributed to its poor dissolution.

Keywords: substandard and falsified medicines, near-infrared chemical imaging, roxithromycin

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Suzuki Y*1, Sugiyama H*2, Kano M*3, Shimono R*4, Shimada G*5, Furukawa R*6, Mano E*7, Motoyama K*8, Koide T, Matsui Y*9, Kurasaki K*10, Takayama I*11, Hikage S*11, Katori N, Kikuchi M*8, Sakai H*12, Matsuda Y*11: Control Strategy and Methods for Continuous Direct Compression Processes.

Asian J Pharm Sci. 2021;16(2):253–62 doi: 10.1016/j.ajps.2020.11.005

We presented a control strategy for tablet manufacturing processes based on continuous direct compression. The work was conducted by the experts of pharmaceutical companies, machine suppliers, academia, and regulatory authority in Japan. Among different items in the process, the component ratio and blended powder content were selected as the items requiring the control method specific to continuous manufacturing different from the conventional batch manufacturing. The control and management of the Loss in Weight (LIW) feeder were deemed the most important, and the Residence Time Distribution (RTD) model were regarded effective for setting the control range and for controlling of the LIW feeder. Based on these ideas, the concept of process control using RTD was summarized. The presented contents can serve as a solid fundament for adopting a new control method of continuous direct compression processes in and beyond the Japanese market.

Keywords: continuous manufacturing, process control, residence time distribution

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Sakai-Kato K*, Yoshida K*, Takechi-Haraya Y, Izutsu K: Physicochemical characterization of liposomes that mimics lipid composition of exosomes for effective intracellular trafficking.

Langmuir. 2020;36:12735-44. doi: 10.1021/acs.langmuir.0c02491

Exosomes mediate communication between cells in the body by the incorporation and transfer of biological materials. To design an artificial liposome, which would mimic the lipid composition and physicochemical characteristics of naturally occurring exosomes, we first studied the physicochemical properties of exosomes secreted from HepG2 cells. The exosome stiffness obtained by atomic force microscopy was moderate. Some liposomes were then fabricated to mimic the representative reported lipid composition of exosomes. Their physicochemical

properties and cellular internalization efficiencies were investigated to optimize the cellular internalization efficiency of the liposomes. A favorable internalization efficiency was obtained by incubating HeLa cells with 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC)/cholesterol (Chol)/1,2-dioleoyl-sn-glycero-3-phospho-l-serine (DOPS) (40/40/20 mol %) liposomes, which have a similar stiffness and zeta potential to exosomes. A dramatic increase in internalization efficiency was demonstrated by adding DOPS to simple DSPC/Chol liposomes. We found that DOPS had a more desirable effect on cellular internalization than its saturated lipid counterpart, 1,2-distearoyl-sn-glycero-3-phospho-l-serine. Furthermore, it was shown that the phosphatidylserine-binding protein, T-cell immunoglobulin mucin protein 4, was largely involved in the intracellular transfer of DSPC/Chol/DOPS liposomes. Thus, DOPS was a key lipid to provide the appropriate stiffness, zeta potential, and membrane surface affinity of the resulting liposome. Our results may help develop efficient drug carriers aiming to internalize active substances into cells.

Keywords: exosome, vesicle stiffness, drug carrier

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Takechi-Haraya Y, Matsuoka M*, Imai H*, Izutsu K, Sakai-Kato K*: Detection of material-derived differences in the stiffness of egg yolk phosphatidylcholine-containing liposomes using atomic force microscopy.

Chemistry and Physics of Lipids. 2020;233:104992. doi: 10.1016/j.chemphyslip.2020.104992

Naturally sourced phospholipids are used in many liposomal pharmaceuticals. The present report describes a method to detect the effects of different egg yolk phosphatidylcholines (EPCs) on liposomal physicochemical properties. Five EPC-containing liposomes were prepared using five different EPCs obtained from different suppliers. There was no significant difference in purity between each EPC. The stiffness of the liposomes was examined via atomic force microscopy (AFM) in relation to the liposomal membrane permeability coefficient of encapsulated calcein after gel filtration, which is indicative of liposomal stability including the release of a hydrophilic drug from a liposome. Although the size of the liposome and the encapsulation efficiency of calcein did not significantly change with the type of EPC used, the liposome stiffness was found to vary depending on the EPC used, and liposomes with a similar stiffness were found to show a similar membrane permeability to calcein. Our results indicate the usefulness of stiffness measurement, using AFM as the analytical method, to detect material-derived differences in EPC-containing liposomes that affect drug release from the liposomes. Because drug release is one of the most important liposomal functions, combining this method with other analytical methods could be useful in selecting material for the development and quality control of EPC-containing liposomes.

Keywords: liposome stiffness, membrane permeability, naturally sourced phospholipid

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Takechi-Haraya Y, Goda Y, Izutsu K, Sakai-Kato K*: Instrument-dependent factors affecting the precision in the atomic force microscopy stiffness measurement of nanoscale liposomes.

Chem Pharm Bull. 2020;68:473-8. doi: 10.1248/cpb.c20-00067

The mechanical strength (stiffness) of liposomes affects their cellular uptake efficiency and drug release in drug delivery processes. We recently developed a tip shape evaluation method for improving the precision of liposome stiffness measurement by quantitative imaging (QI)-mode atomic force microscopy (AFM). The present study applied our method to the widely-used AFM instruments equipped for intermittent contact (IC)-mode force curve measurements, and examined instrument-dependent factors that affect the liposome stiffness measurements. We demonstrated that the evaluation of the tip shape for cantilever selection can be applicable to the IC mode as well as the QI mode. With the cantilever selection, the improved precision of the liposome stiffness was obtained when the stiffness of each liposome was determined from the slope in the force-deformation curve by the IC-mode force curve measurement. Further, the stiffness values were found to be similar to that measured by QI-mode measurements. These results indicate that our developed method can be widely used via IC-mode force curve measurements

as well as via QI mode. It was also revealed that spatial drift of the cantilever position was instrument-dependent factors which could affect the precision of liposome stiffness measurements in the case of IC-mode force curve measurement. Therefore, in case of stiffness measurement by IC-mode force curve measurement, it is vital to obtain force-deformation curves immediately after imaging a liposome for the precise stiffness measurement of liposomes. These findings will promote the usage of the AFM stiffness measurement method for the characterization of lipid nanoparticle-based drug delivery systems.

Keywords: atomic force microscopy, instrument difference, liposome stiffness

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Ohgita T[*1], Takechi-Haraya Y, Okada K[*1], Matsui S[*1], Takeuchi M[*1], Saito C[*2], Nishitsuji K[*3], Uchimura K[*4], Kawano R[*2], Hasegawa K[*1], Sakai-Kato K[*5], Akaji K[*1], Izutsu K, Saito H[*1]: Enhancement of direct membrane penetration of arginine-rich peptides by polyproline II helix structure.

Biochim Biophys Acta Biomembr. 2020;1862:183403. doi: 10.1016/j.bbamem.2020.183403

The left-handed, extended polyproline II (PPII) helix is a unique secondary structure which potently modulates peptide/protein functions through its constraint conformation. To investigate the effect of PPII helix on the direct cell membrane penetration of arginine-rich peptides, we designed a polyproline-containing arginine-rich peptide P9R7W (PPPPPPPPPRRRRRRRW) by introducing nine proline residues into a linear R7W (RRRRRRRW) peptide. Circular dichroism spectroscopy showed that P9R7W has the PPII helix structure in solution whereas R7W is predominantly in random coil structure. Tryptophan fluorescence measurements demonstrated that P9R7W binds to negatively charged lipid vesicles with similar affinity to R7W, in which there was no significant change in the PPII helix structure. Flow cytometry and confocal laser scanning microscopy analyses showed that P9R7W has an ability to penetrate into CHO-K1 cells more efficiently compared to R7W with no cytotoxicity. Consistently, a channel current analysis unveiled that P9R7W penetrates planar lipid bilayer membranes more efficiently than R7W without significant membrane perturbation. Our results indicate that the PPII helix structure can enhance the membrane penetration efficiency of arginine-rich peptides without lipid membrane perturbation.

Keywords: arginine-rich peptide, polyproline II helix, cell membrane penetration

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Sakai-Kato K[*1], Takechi-Haraya Y, Chida T[*2], Okazaki M[*3], Kozaki M[*2]: Robust nanoparticle morphology and size analysis by atomic force microscopy for standardization.

Chem Pharm Bull. 2020;68:791-6. doi: 10.1248/cpb.c20-00311

Because of the complexity of nanomedicines, analysis of their morphology and size has attracted considerable attention both from researchers and regulatory agencies. The atomic force microscope (AFM) has emerged as a powerful tool because it can provide detailed morphological characteristics of nanoparticles both in the air and in aqueous medium. However, to our knowledge, AFM methods for nanomedicines have yet to be standardized or be listed in any pharmacopeias. To assess the applicability of standardization of AFM, in this study, we aimed to identify robust conditions for assessing the morphology and size of nanoparticles based on a polystyrene nanoparticle certified reference material standard. The spring constant of the cantilever did not affect the size of the nanoparticles but needed to be optimized depending on the measurement conditions. The size analysis method of the obtained images affected the results of the analyzed size values. The results analyzed by cross-sectional line profiling were independent of the measurement conditions and gave similar results to those from dynamic light scattering. It was indicated that approximately 100 particles are required for a representative measurement. Under the optimized conditions, there were no significant inter-instrument differences in the analyzed size values of polystyrene nanoparticles both in air and under

aqueous conditions.

Keywords: atomic force microscopy, size measurement, standardization

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Kosuge H*1, Nagatoishi S*1, Kiyoshi M, Ishii-Watabe A, Tanaka T*2, Terao Y*2, Oe S*2, Ide T*2, Tsumoto K*1: Highly Sensitive HPLC Analysis and Biophysical Characterization of N-glycans of IgG-Fc Domain in Comparison Between CHO and 293 Cells Using FcγRIIIa Ligand.

Biotechnol Prog. 2020:e3016. doi: 10.1002/btpr.3016

Quality control of monoclonal antibodies is challenging due in part to the diversity of post-translational modifications present. The regulation of the N-glycans of IgG-Fc domain is one of the key factors to maintain the safety and efficacy of antibody drugs. The FcγRIIIa affinity column is an attractive tool for the precise analysis of the N-glycans in IgG-Fc domain. We used the mutant FcγRIIIa, which is produced in Escherichia coli and is therefore not glycosylated, as an affinity reagent to analyze the N-glycans of monoclonal antibodies expressed in Expi293 and ExpiCHO cells. The monoclonal antibodies expressed in these cells showed very different chromatograms, because of differences in terminal galactose residues on the IgG-Fc domains. We also carried out kinetic and thermodynamic analyses to understand the interaction between monoclonal antibodies and the mutant FcγRIIIa. Expi293 cell-derived monoclonal antibodies had higher affinity for the mutant FcγRIIIa than those derived from ExpiCHO cells, due to slower off rates and lower binding entropy loss. Collectively, our results suggest that the FcγRIIIa column can be used to analyze the glycosylation of antibodies rapidly and specifically.

Keywords: FcγRIIIa column, N-glycosylation, antibody drug

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Hyuga S*1, Hyuga M, Amakura Y*2, Yang J*3, Mori E*1, Hakamatsuka T, Goda Y, Odaguchi H*1, Hanawa T*1: Effect of Ephedra Herb on Erlotinib Resistance in c-Met-Overexpressing Non-Small-Cell Lung Cancer Cell Line, H1993, through Promotion of Endocytosis and Degradation of c-Met.

Evid Based Complement Alternat Med. 2020;2020: 7184129. doi: 10.1155/2020/7184129

Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (EGFR-TKIs) are used to treat non-small-cell lung cancer (NSCLC), harboring an EGFR-activating mutation. However, acquired resistance to these treatments emerges after a few years. One of causes of resistance to EGFR-TKIs is a high level of c-Met amplification or c-Met protein overexpression/hyperactivation. Therefore, combination therapy with EGFR-TKIs and a c-Met inhibitor is thought to be effective treatment for patients with NSCLC resistance carrying c-Met amplification and/or protein hyperactivation. Ephedra Herb is a crude drug and is used in Japan as a component in many Kampo formulae. We previously reported that Ephedra Herb extract (EHE) inhibits HGF-induced phosphorylation of c-Met by preventing c-Met tyrosine kinase activity. Thus, we investigated the combination effect of EHE and erlotinib, an EGFR-TKI, on growth of H1993 cells, an erlotinib-resistant NSCLC cell line with overexpression of c-Met. The EHE and erlotinib combination proved to be effective in suppression of the growth of H1993 xenograft tumors and on inhibition of proliferation of H1993 cells, suggesting that EHE is effective in rescuing NSCLC cells from erlotinib resistance. Moreover, EHE not only inhibited the phosphorylation of c-Met, but also downregulated the expression of c-Met by facilitating clathrin-mediated endocytosis and lysosomal degradation of c-Met. EHE also promoted downregulation of the expression of EGFR and phosphorylation of EGFR. Ephedrine alkaloids-free Ephedra Herb extract (EFE) had the same effects as EHE, and the 40% MeOH fraction from EFE, which mainly contained the high-molecular mass condensed tannins, decreased the expression levels of c-Met, pMet, EGFR, and pEGFR to almost the same level as EFE. These results suggest that recovery from resistance to erlotinib by EHE is derived from the high-molecular mass condensed tannins and that EHE may be suitable for treatment of c-Met-overexpressing NSCLC with resistance to EGFR-TKIs.

Keywords: c-Met, EGFR, erlotinib resistance

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Kiyoshi M, Tatematsu K*, Tada M, Sezutsu H*, Shibata H, Ishii-Watabe A：Structural insight and stability of TNFR-Fc fusion protein (Etanercept) produced by Using transgenic silkworms.
J Biochem. 2020 Aug 7; mvaa092. doi: 10.1093/jb/mvaa092

Therapeutic proteins expressed using transgenic animals have been of great interest for several years. Especially, transgenic silkworm has been studied intensively because of its ease in handling, low-cost, high-yield and unique glycosylation patterns. However, the physicochemical property of the therapeutic protein expressed in transgenic silkworm remains elusive. Here, we constructed an expression system for the TNFR-Fc fusion protein (Etanercept) using transgenic silkworm. The TNFR-Fc fusion protein was employed to N-glycan analysis, which revealed an increased amount of afucosylated protein. Evidence from surface plasmon resonance analysis showed that the TNFR-Fc fusion protein exhibit increased binding affinity for Fcγ receptor IIIa and FcRn compared to the commercial Etanercept, emphasizing the profit of expression system using transgenic silkworm. We have further discussed the comparison of higher order structure, thermal stability and aggregation of the TNFR-Fc fusion protein.

Keywords: Fc fusion protein, glycosylation, physicochemical property

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Tajiri-Tsukada M, Hashii N, Ishii-Watabe A：Establishment of a highly precise multi-attribute method for the characterization and quality control of therapeutic monoclonal antibodies.
Bioengineered. 2020;11:984-1000. doi: 10.1080/21655979.2020.1814683

The multi-attribute method (MAM) has garnered attention as a new quality control method of therapeutic monoclonal antibodies (mAbs). MAM analysis allows multiple relative quantifications of several structural attributes of therapeutic mAbs; however, some issues remain to be addressed in its procedures especially for sample preparation. The goal of this study was to optimize the sample preparation method for MAM analysis of mAbs. Using a model mAb, we compared five sample preparation methods based on sequence coverage, peptide redundancy, missed cleavage and chemical deamidation. It was found that low pH buffer and short digestion time reduced artificial deamidation. The desalting process after carboxymethylation was essential to obtaining high sequence coverage by a short digestion time. The generation of missed cleavage peptides was also improved by using a trypsin/lysyl endopeptidase (Lys-C) mixture. Next, we evaluated the usefulness of our method as a part of MAM analysis. Finally, 17 glycopeptides, 2 deamidated peptides and N- and C-terminal peptides of the heavy chain were successfully monitored with acceptable mass accuracy and coefficient of variation (CV, %) of the relative peak area. On the other hand, 4 oxidated peptides indicated the unavoidable slightly higher inter-assay CV (%) of the peak area ratio due to the instability in the MS sample solution. Collectively, we demonstrated that our method was applicable as an easy and reliable sample preparation method for MAM analysis, and the variation in the relative peak area could be influenced by the modification type rather than by the amount of each peptide.

Keywords: multi-attribute method, monoclonal antibody, peptide mapping

Kiyoshi M, Tada M, Shibata H, Aoyama M, Ishii-Watabe A: Characterization of Aggregated Antibody-Silicone Oil Complexes; From Perspectives of Morphology, 3D Image, and Fcγ Receptor Activation.
J Pharm. Sci. 2020;S0022-3549(20):30614-6. doi: 10.1016/j.xphs.2020.10.022

Pre-filled syringes (PFS) have been in widespread use as an administration device for therapeutic antibodies in recent decades. Generally, the inner barrel and syringe of PFS are coated with silicone oil (SO) for lubrication. Multiple studies have focused on the fact that the SO adsorbs denatured antibody

molecules, and induces antibody aggregation. Aggregated antibodies are recognized as a potential risk for evoking immunogenic responses in patients. The characteristics of the aggregated antibody-SO complexes, including their concentration, population, shape, three-dimensional (3D) image, and Fcγ Receptors (FcγRs) activation have been obscurely acknowledged so far. In the present work, we prepared aggregated antibody-SO complexes by agitation and analyzed using multifaceted techniques such as flow imaging, confocal fluorescence microscopy, and cell-based assays for FcγRs activation. The results emphasized that the SO accelerates the increase in sub-visible particles and antibody aggregation. The confocal fluorescence microscopy analysis revealed the high-resolution 3D images of aggregated antibody-SO complexes. The FcγRs reporter cell assay clarified that the pre-mixed and agitated Ab + SO have higher FcγRs activation capability compared to the agitated Ab. Overall, this study advances the view that SO has an effect to increase the risk of agitation-induced aggregated antibody particles.

Keywords: biopharmaceuticals, therapeutic antibody, silicone oil

Ueda K[*1], Shimizu M[*1], Ohashi A[*1], Murata D[*1], Suzuki T, Kobayashi N[*1], Baba J[*1], Takeuchi T[*2], Shiga Y[*1], Nakamura M[*1], Kagaya S[*3], Sato A[*1]: Albumin fusion at the N-terminus or C-terminus of human lactoferrin leads to improved pharmacokinetics and anti-proliferative effects on cancer cell lines.

Eur J Pharm Sci. 2020;155:105551. doi: 10.1016/j.ejps.2020.105551

Human lactoferrin (hLF), a soluble factor of the innate immune system, exhibits various biological functions and therefore has potential as a therapeutic protein. However, the clinical applications of hLF are limited by its low stability in blood. We therefore attempted to resolve this by producing recombinant hLF fused to human serum albumin (HSA). Two HSA-fused hLFs with different fusion orientations (hLF-HSA and HSA-hLF) were produced in Chinese hamster ovary (CHO) DG44 cells. hLF-HSA revealed higher thermal stability, resistance to peptic degradation, and stability during the process of cellular uptake and release in an intestinal enterocyte model (Caco-2 cells) than HSA-hLF. The lower stability of HSA-hLF is presumably due to the steric hindrance imposed by HSA fusion to the N-terminus of hLF. Both HSA fusion proteins, especially HSA-hLF, displayed improved pharmacokinetic properties despite the lower protein stability of HSA-hLF. hLF-HSA and HSA-hLF exhibited approximately 3.3- and 20.7-fold longer half-lives (64.0 and 403.6 min), respectively, than holo-rhLF (19.5 min). Both HSA fusion proteins were found to exert enhanced growth inhibition effects on cancer cells in vitro, but not normal cells. Their enhanced growth inhibitory activities were considered to be due to the synergetic effects of hLF and HSA because hLF alone or HSA alone failed to exert such an effect. Altogether, Fusion of HSA to hLF yielded superior pharmacokinetics and anti-proliferative activities against cancer cells. HSA-fused hLF is a novel candidate for further application of hLF as biopharmaceuticals for intravenous administration.

Keywords: cancer cell growth inhibition, fusion protein, human lactoferrin

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森本和滋，日向昌司，石井明子

バイオ医薬品の品質評価技術の進歩と国際調和：国立医薬品食品衛生研究所生物薬品部30年の歩みに焦点を絞って.

薬史学雑誌 2020;55 (2):169-178. doi: 10.34531/jjhp.55.2_169

国立医薬品食品衛生研究所・生物薬品部の30年の歩みを，バイオ医薬品の承認，品質評価法の研究開発，国際調和の視点から調べた．生物薬品部の歴史は，国立医薬品食品衛生研究所報告より，バイオ医薬品の承認情報は，生物薬品部のウェブサイトより，またICHガイドラインの情報は，PMDAのウェブサイトより収集した．第1期（1989–1998）20品目のバイオ医薬品が承認された．従来動物を用いたバイオアッセイの代替法としてのHPLC法の検討，蛍光体支援糖鎖電気泳動法を用いてr-hEPO糖鎖パターンを解析し，分子多様性を解析する方法を開発した．ICHガイドラインQ5BとQ5Cが調和した．1993年には、三局方間（欧州薬局方、日本薬局方，米国薬局方）オープン会議，バイオ医薬品規格基

準のハーモナイゼーションが開催された．第２期（1999–2008）35品目のバイオ医薬品が承認された．MS例えばLC/ESI-MSを用いて，r-hEPOの糖鎖構造を解析する方法を開発した．ICH 品質ガイドライン，Q5A（R1），Q5D，Q6B，Q5Eが調和した．第３期（2009–2018）82品目のバイオ医薬品が承認された．抗体医薬品の多様性をLC/MSカラムスイッチ法で調べる方法を開発した．ICHガイドライン「医薬品のライフスタイルマネジメント」Q12が合意に達した．わが国のバイオ医薬品の特性解析と品質管理の最近のトピックス，たとえばモノクローナル抗体についても検討した．

Keywords：バイオ医薬品の承認，バイオ医薬品の品質評価法，ICH

Nakamura H[*1], Kiyoshi M, Anraku M[*1], Hashii N, Oda-Ueda N[*1], Ueda T[*2] and Ohkuri T[*1]: Glycosylation decreases aggregation and immunogenicity of adalimumab Fab secreted from Pichia pastoris.

J Biochem. 2021. doi: 10.1093/jb/mvaa116

Glycoengineering of therapeutic proteins has been applied to improve the clinical efficacy of several therapeutics. Here, we examined the effect of glycosylation on the properties of the Fab of the therapeutic antibody, adalimumab. An N-glycosylation site was introduced at position 178 of the H-chain constant region of adalimumab Fab through site-directed mutagenesis (H: L178N Fab), and the H: L178N Fab was produced in Pichia pastoris. Expressed mutant Fab contained long and short glycan chains (L-glyco Fab and S-glyco Fab, respectively). Under the condition of aggregation of Fab upon pH shift-induced stress, both of L-glyco Fab and S-glyco Fab were less prone to aggregation, with L-glyco Fab suppressing aggregation more effectively than the S-glyco Fab. Moreover, the comparison of the antigenicity of glycosylated and wild-type Fabs in mice revealed that glycosylation resulted in the suppression of antigenicity. Analysis of the pharmacokinetic behavior of the Fab, L-glyco Fab, and S-glyco Fab indicated that the half-lives of glycosylated Fabs in the rats were shorter than that of wild-type Fab, with L-glyco Fab having a shorter half-life than S-glyco Fab. Thus, we demonstrated that the glycan chain influences Fab aggregation and immunogenicity, and glycosylation reduces the elimination half-life in vivo.

Keywords: Fab, aggregation, glycoengineering

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Hashii N, Tousaka Y, Arai K[*1], Enoki Y[*2], Fukuda S[*2], Goda R[*3], Inoue N[*4], Kawabata M[*2], Murata K[*5], Nakatsuji M[*4], Okuzono T[*6], Shigeyama T[*5], Tachiki H[*4], Yamaguchi T[*5], Yamane S[*6], Yamaoka M[*4], Saito Y, Ishii-Watabe A: Bioanalysis of therapeutic monoclonal antibody by peptide adsorption-controlled LC-MS.

Bioanalysis. 2021;13:265-276. doi: 10.4155/bio-2020-0262

Aim: We aimed to develop an easy, low-cost and versatile mass spectrometric method for the bioanalysis of a therapeutic monoclonal antibody (mAb) in human serum that employs peptide adsorption-controlled (PAC)-LC/MS using selected reaction monitoring mode (LC-MS/MS-SRM). Materials & methods: Rituximab was used as a model mAb. To apply the method to human serum samples, a peptide of the complementarity-determining region was selected as the surrogate peptide. The usefulness of PAC-LC-MS/MS-SRM was evaluated by a collaborative study. Results: The calibration curve ranged from 0.5 (or 1.0) to 1000.0 µg/ml. The selectivity, linearity, accuracy and precision met the predefined acceptance criteria. Conclusion: Our method could be a useful bioanalytical method for the quantification of mAbs in clinical samples.

Keywords: bioanalysis, human serum, peptide adsorption-controlled LC/MS

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袴塚高志，鎌倉浩之，渡辺淳子*，香取征典*，松本和弘*，石丸順之*，諸田隆*，合田幸広：葛根湯エキス顆粒および葛根湯エキス錠剤の生物学的同等性試験．

生薬学雑誌 2020;74:89-97.

Dry extract preparations of Kampo medicines for prescription were approved for use approximately

40 years ago in Japan. Presently, most Kampo medicines are prepared in the form of granules with a few being prepared as tablets or capsules. Granule formulations are generally unsuitable for the elderly due to their bulky nature. Although patients and Kampo manufacturers have expressed a need for the introduction of more acceptable granule alternatives, their introduction has been a challenge due to the lack of guidelines based on bioequivalence evaluations for medicines that include multiple chemical components. For resolving this issue, the researchers at the National Institute of Health Sciences initiated a study in 2009 funded by the Ministry of Health, Labour and Welfare. Several ingredients in Kampo extract products and corresponding standard decoctions were detectable and measurable in human plasma, and some compounds have been reported to be promising candidates for application in bioequivalence evaluations of Kampo formulations. The purpose of the present study was to investigate the potential to assess bioequivalence between kakkonto extract granules and tablets on the basis of the "Guidelines for Bioequivalence Testing of Generic Drugs (partial revision, PFSB/ELD Notification No. 0229010 dated February 29, 2012)."

We investigated the pharmacokinetics of ephedrine and pseudoephedrine, which are ingredients derived from Ephedra Herba in Kakkonto formulations, following the oral administration of Kakkonto extract granules (one pack) and Kakkonto extract tablets (eight tablets). The study was conducted as a two-group, two-period, and open-label crossover study in healthy Japanese volunteers. The plasma concentrations of ephedrine and pseudoephedrine following the administration of the drugs were measured using liquid chromatography with tandem mass spectrometry. Subsequently, we calculated their pharmacokinetic parameters and evaluated their bioequivalence. Analysis of variance using the area under the plasma concentration time curve (AUC) and the maximum plasma concentration (C_{max}) of both ingredients revealed that while AUC indicated bioequivalence, C_{max} values were significantly different. Plasma concentration levels in both formulations were similar in most volunteers and differed among some volunteers, which was attributed to a high number of tablets per dose as opposed to intra-individual variation. We concluded that ephedrine and pseudoephedrine in Kakkonto extracts are good marker compounds for the evaluation of bioequivalence in different forms of Kakkonto products.

Our results suggest that the marker compounds exhibiting similarity in pharmacokinetic parameters following the administration of Kampo extract granules and the corresponding standard decoction could be applied as markers for the evaluation of bioequivalence between already-approved Kampo extract granules and novel Kampo products based on the same extract as that of granules.

Keywords: bioequivalence, Hachimijiogan, benzoylmesaconine

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Batsukh Z[*1], Toume K[*1], Javzan B[*2], Kazuma K[*1], Cai SQ[*3], Hayashi S[*4], Kawahara N[*4], Maruyama T, Komatsu K[*1]: Metabolomic profiling of Saposhnikoviae Radix from Mongolia by LC-IT-TOF-MS/MS and multivariate statistical analysis.

J. Nat. Med. 2020;74:170-188. doi: 10.1007/s11418-019-01361-0

Saposhnikoviae Radix (SR) is a commonly used crude drug that is obtained from the root and rhizome of *Saposhnikovia divaricata* which is distributed throughout China, Korea, Mongolia, and Russia. To evaluate the quality of Mongolian *S. divaricata*, metabolomic profiling of 43 plant specimens from different regions of Mongolia, as well as 8 SR samples and 2 plant specimens from China, were conducted by liquid chromatography–ion-trap–time-of-flight–mass spectrometer (LC– IT–TOF–MS). LC–MS profiles of the specimens showed uniformity and 30 compounds were tentatively identified, including 13 chromones and 17 coumarins. Among them, 16 compounds were isolated and unambiguously verified by comparing them with the spectroscopic data of standard compounds. Orthogonal partial least squares–discriminant analysis (OPLS–DA) based on LC–MS data from 7 Mongolian specimens and 8 Chinese SR samples as well as 2 plant specimens revealed that these 2 groups were clearly distinguishable and that Mongolian specimens were characterized by an abundance of prim-*O*-glucosylcimifugin (**1**). Moreover, the OPLS–DA of the Mongolian specimens showed

that they can be discriminated by their growing regions based on the content of 8 chromones. The total content of dihydrofurochromones **1**–**3** was relatively higher in the specimens from Khalkhgol in the far eastern part of Mongolia, while contents of **10**, **11**, **15**, and **16** were higher in those from Holonbuir in the eastern part. Based on this research, the roots of *S. divaricata* from Mongolia have potential as a new resource of SR in Kampo medicine.

Keywords: Saposhnikoviae Radix, Mongolia, multivariate statistical analysis

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Wang Z*1, Okutsu K*1, Futagami T*1, Yoshizaki Y*1, Tamaki H*1, Maruyama T, Toume K*2, Komatsu K*2, Hashimoto F*1, Takamine K*1: Microbial community structure and chemical constituents in *Shinkiku*, a fermented crude drug used in Kampo medicine.

Frontiers in Nutrition. 2020;7:115. doi: 10.3389/fnut.2020.00115

Shinkiku (Massa Medicata Fermentata) is a traditional crude drug used to treat anorexia and dyspepsia of elder patients in east Asia. *Shinkiku* is generally prepared by the microbial fermentation of wheat and herbs. *Shinkiku* is also used in Japanese Kampo medicine as a component of 半夏白朮天麻湯 (Hangebyakujutsutemmato). However, the quality of *shinkiku* varies by manufacture because there are no reference standards to control the quality of medicinal *shinkiku*. Thus, we aim to characterize the quality of various commercially available *shinkiku* by chemical and microbial analysis. We collected 13 *shinkiku* products manufactured in China and Korea and investigated the microbial structure and chemical constituents. Amplicon sequence analysis revealed that *Aspergillus* sp. was common microorganism in *shinkiku* products. Digestive enzymes（α-amylase, protease, and lipase), organic acids (ferulic acid, citric acid, lactic acid, and acetic acid), and 39 volatile compounds were commonly found in *shinkiku* products. Although there were some commonalities in *shinkiku* products, microbial and chemical characteristic considerably differed as per the manufacturer. *Aspergillus* sp. was predominant in Korean products, and Korean products showed higher enzyme activities than Chinese products. Meanwhile, *Bacillus* sp. was commonly detected in Chinese *shinkiku*, and ferulic acid was higher in Chinese products. Principal component analysis based on the GC-MS peak area of the volatiles also clearly distinguished *shinkiku* products manufactured in China from those in Korea. Chinese products contained higher amounts of benzaldehyde and anethole than Korean ones. Korean products were further separated into two groups: one with relatively higher linalool and terpinen-4-ol and another with higher hexanoic acid and 1-octen-3-ol. Thus, our study revealed the commonality and diversity of commercial *shinkiku* products, in which the commonalities can possibly be the reference standard for quality control of *shinkiku*, and the diversity suggested the importance of microbial management to stabilize the quality of *shinkiku*.

Keywords: shinkiku, *Aspergillus* sp., ferulic acid

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Batsukh Z*1, Toume K*1, Javzan B*2, Kazuma K*1, Cai SQ*3, Hayashi S*4, Atsumi T*5, Yoshitomi T, Uchiyama N, Maruyama T, Kawahara N*4, Komatsu K*1: Characterization of metabolites in *Saposhnikovia divaricata* root from Mongolia.

J. Nat. Med. 2021;75:11-27. doi: 10.1007/s11418-020-01430-9

Saposhnikoviae Radix (SR), derived from the dried root and rhizome of *Saposhnikovia divaricata*, is a popular crude drug used in traditional Chinese and Japanese medicine. To evaluate the metabolites of *S. divaricata* roots from Mongolia and to investigate their geographical variation, we developed the HPLC method, determined the contents of 9 chromones and 4 coumarins, and conducted multivariate statistical analysis. All Mongolian specimens contained prim-*O*-glucosylcimifugin (**1**) and 4'-*O*-β-D-glucosyl-5-*O*-methylvisamminol (**3**), and their total amount

(5.04–25.06 mg/g) exceeded the criterion assigned in the Chinese Pharmacopoeia. Moreover, the content of **1** (3.98–20.79 mg/g) was significantly higher in the Mongolian specimens than in Chinese SR samples. The specimens from Norovlin showed the highest contents of **1** and **3**. The total levels of dihydropyranochromones were higher in the specimens from Bayan-Uul. The orthogonal partial least squares-discriminant analysis revealed that the Mongolian specimens tended to be separated into three groups based on growing regions, in which several chromones contributed to each distribution. Furthermore, ^{1}H NMR analysis revealed that Mongolian specimens had less amount of sucrose and a substantial amount of polyacetylenes. Thus, in this study, the chemical characteristics of Mongolian *S. divaricata* specimens were clarified and it was found that the specimens from the northeast part of Mongolia, including Norovlin, had the superior properties due to higher amounts of major chromones.

Keywords: Saposhnikoviae Radix, Mongolia, multivariate statistical analysis

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Yoshitomi T, Wakana D*1, Uchiyama N, Tsujimoto T, Kawano N*2, Yokokura T*3, Yamamoto Y*4, Fuchino H*2, Hakamatsuka T, Komatsu K*5, Kawahara N*2, Maruyama T: Identification of the responsible compounds for the discrimination of Saposhnikovia root from Peucedanum ledebourielloides root using LC-HRMS metabolome.

J. Nat. Med. 2020;74:550-560. doi: 10.1007/s11418-020-01409-6

Previously, we established a ^{1}H NMR metabolomics method using reversed-phase solid-phase extraction column (RP- SPEC), and succeeded in distinguishing wild from cultivated samples of Saposhnikoviae radix (SR), and between SR and its substitute, *Peucedanum ledebourielloides* root (PR). Herein, we performed LC-HR/MS metabolomics using fractions obtained via RP-SPEC to identify characteristic components of SR and PR. One and three characteristic components were respectively found for SR and PR; these components were isolated with their *m/z* values and retention times as a guide. The characteristic component of SR was identified as 4'-*O*-β-D-glucosyl-5-*O*-methylvisamminol (**1**), an indicator component used to identify SR in the Japanese Pharmacopoeia. In contrast, the characteristic components of PR were identified as xanthalin (**2**), 4'-*O*-β-D-apiosyl (1→6)-β-D-glucosyl-5-*O*-methylvisamminol (**3**), and 3'-*O*-β-D-apiosyl (1→6)-β-D-glucosylhamaudol (**4**) based on spectroscopic data such as 1D- and 2D-NMR, MS, and specific optical rotation. Among them, **4** is a novel compound. For the correlation between the NMR metabolomics results in the present and our previous report, only **1** and **2** were found to correlate with the chemical shifts, and the other compounds had no correlation. As the chemical shifts for compounds **1**, **3**, and **4** were similar to each other, especially for the aglycone moiety, they could not be distinguished because of the sensitivity and resolution of ^{1}H NMR. Accordingly, combining NMR and LC/MS metabolomics with their different advantages is considered useful for metabolomics of natural products. The series of methods used in our reports could aid in quality evaluations of natural products and surveying of marker components.

Keywords: ^{1}H NMR-based metabolomics, LC-HR/MS metabolomics, Saposhnikoviae Radix

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Iijima L*1, Kishimoto S*2, Ohmiya A*2, Yagi M*2, Okamoto E*1, Miyahara T*1,3, Tsujimoto T*1, Ozeki Y*1, Uchiyama N, Hakamatsuka T, Kouno T*4, Cano EA*5, Shimizu M*6, Nishihara M*6: Esterified carotenoids are synthesized in petals of carnation (*Dianthus caryophyllus*) and accumulate in

differentiated chromoplasts.

Sci. Rep. 2020;10:15256. doi: 10.1038/s41598-020-72078-4

Here, we identified a carnation cultivar with pale yellow flowers that accumulated carotenoids in petals. Additionally, some xanthophyll compounds were esterified, as is the case for yellow flowers in other plant species. Ultrastructural analysis showed that chromoplasts with numerous plastoglobules, in which flower-specific carotenoids accumulate, were present in the pale yellow petals. RNA-seq and RT-qPCR analyses indicated that the expression levels of genes for carotenoid biosynthesis and esterification in pale yellow and pink petals (that accumulate small amounts of carotenoids) were similar or lower than in green petals (that accumulate substantial amounts of carotenoids) and white petals (that accumulate extremely low levels of carotenoids). Pale yellow and pink petals had a considerably lower level of expression of genes for carotenoid degradation than white petals, suggesting that reduced degradation activity caused accumulation of carotenoids. Our results indicate that some carnation cultivars can synthesize and accumulate esterified carotenoids.

Keywords: carnation, *Dianthus caryophyllus*, esterified carotenoids

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Sawamoto A[*1], Kanazaki A[*1], Amakura Y[*2], Yoshimura M[*2], Masumoto N, Uchiyama N, Hakamatsuka T, Okuyama S[*1], Furukawa Y[*1], Nakajima M[*1]: Cynandione A induces adipogenesis and beige adipocyte-related phenotype in 3T3-L1 cells.

Phytochem. Lett. 2020;39:84-89. doi: 10.1016/j.phytol.2020.07.011

Cynandione A (CA) is a major ingredient of Cynanchum wilfordii. Here, we report a new function of CA in adipogenesis in 3T3-L1 cells, a preadipocyte cell line. During adipogenic differentiation in 3T3-L1 cells, CA boosted the process by enhancing the expression of key adipogenic transcription factors (Pparg and C/ebpa), brown adipocyte-related genes (Prdm16, Pgc-1a, Cidea, and Ucp1), and beige adipocyte-related genes (Tbx1 and Cited1). Additionally, CA increased mitochondrial mass and expression levels of mitochondria-related genes (Sirt3 and Tfam) in the cells. These results suggest that CA induces adipogenesis and beige adipocyte-related phenotype in adipocyte lineage cells.

Keywords: Cynandione A, beige adipocyte, adipogenesis

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新井玲子，政田さやか，田中誠司，袴塚高志，内山奈穂子：指定成分であるコレウス・フォルスコリーを含む健康食品の定性及び定量分析.

日本食品化学学会誌 2020;27:84-92. doi:10.18891/jjfcs.27.2_84

In 2020, the government of Japan has designated *Coleus forskohlii* as “an ingredient calling for special attention”. In this study, we established a specific TLC identification method and a quantitative HPLC assay, and evaluated the quality and quantity of 14 health food products. For TLC identification and for a cleaner analysis method, we modified the USP method in which toluene is used as the TLC eluent. For HPLC quantification of forskolin in *C. forskohlii*-containing products, we developed a simpler and more sensitive method based on the USP monograph. Surprisingly, the forskolin content in health food products varied greatly and ranged up to 300 fold (0.35–120.10 mg/day). These developed methods would be useful for the qualitative and quantitative evaluations of health food products containing *C. forskohlii*.

Keywords: ingredient calling for special attention, *Coleus forskohlii*, health food product

Uchiyama N, Hosoe J, Miura T[*1], Sugimoto N, Ishizuki K, Yamada Y[*1], Iwamoto Y[*1], Suematsu T[*2], Komatsu T[*2], Maruyama T[*3], Igarashi Y[*3], Higano T[*4], Shimada N[*5], Goda Y: Determination of absolute purities of hydroscopic substances by quantitative NMR analysis for the standardization of quantitative

reagents in the Japanese Pharmacopoeia (Part 2).
Chem. Pharm. Bull. 2021;69:26-31. doi: 10.1248/cpb.c20-00296

In this study, typical and optimal conditions that affect the determination of the purity of ginsenoside Rb1 (GRB1), saikosaponin a (SSA), and barbaloin (BB) (*i.e.*, hygroscopic reagents) by quantitative NMR (qNMR) were examined. First, the effect of humidity before and during weighing on the purity of commercial GRB1, with a purity value determined by qNMR, was examined. The results showed the importance afore-mentioned. The results of SSA, which is relatively unstable in the dissolved state, suggested that the standardization of humidity control before and during weighing for a specific time provides a practical approach for hygroscopic products. In regard to BB, its humidity control for a specific time, only before weighing, is enough for a reproducible purity determination.

Keywords: quantitative NMR, marker compound, crude drug

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Chem. Pharm. Bull. 2021;69:118-123. doi: 10.1248/cpb.c20-00728.

We examined the absolute purity determination of a hygroscopic substance, indocyanine green (ICG), listed in the Japanese Pharmaceutical Codex 2002, using quantitative NMR (qNMR) for standardization by focusing on the adaptation of ICG to Japanese Pharmacopoeia (JP). The purity of ICG, as an official non-Pharmacopoeial reference standard (non-PRS), had high variation (86.12 ± 2.70%) when preparing qNMR samples under non-controlled humidity (a conventional method). Additionally, residual ethanol (0.26 ± 0.11%) was observed in the non-PRS ICG. Next, the purity of non-PRS ICG was determined via qNMR when preparing samples under controlled humidity using a saturated sodium bromide solution. The purity was 84.19 ± 0.47% with a lower variation than that under non-controlled humidity. Moreover, ethanol signal almost disappeared. We estimated that residual ethanol in non-PRS ICG was replaced with water under controlled humidity. Subsequently, qNMR analysis was performed when preparing samples under controlled humidity in a constant temperature and humidity box. It showed excellent results with the lowest variation (82.26 ± 0.19%).

Keywords: quantitative NMR, hygroscopic substance, humidity control

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Hirasawa Y*1, Agawa-Kakimoto M*1, Zaima K*2, Uchiyama N, Goda Y., Morita H*1: Complanadine F, a novel dimeric alkaloid from *Lycopodium complanatum*.
J. Nat. Med. 2021;75:403-407. doi: 10.1007/s11418-020-01476-9

A novel Lycopodium alkaloid, complanadine F (1), seco-complanadine A type was isolated from the club moss *Lycopodium complanatum*. The planar structure and relative configuration were elucidated based on spectroscopic data.

Keywords: *Lycopodium complanatum*, Complanadine A, Complanadine F

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Zhou T*1,2, Hirayama Y*2, Tsunematsu Y*2, Suzuki N*2, Tanaka S, Uchiyama N, Goda Y, Yoshikawa Y*3, Iwashita Y*4, Sato M*2, Miyoshi N*5, Mutoh M*6, Ishikawa H*6, Sugimura H*4, Wakabayashi K*5, Watanabe K*1,2: Isolation of new colibactin metabolites from wild-type *Escherichia coli* and *in situ* trapping of a mature colibactin derivative.
J. Am. Chem. Soc. 2021;143:5526-5533. doi:10.1021/jacs.1c01495

Colibactin is a polyketide-nonribosomal peptide hybrid secondary metabolite that can form interstrand cross-links in double-stranded DNA. Colibactin-producing *Escherichia coli* has also been linked to colorectal oncogenesis. Thus, there is a strong interest in understanding the role colibactin may play in oncogenesis. Here, using the high-colibactin-producing wild-type *E. coli* strain we isolated from a clinical sample with the activity-based fluorescent probe we developed earlier, we were able to identify colibactin 770, which was recently identified and proposed as the complete form of colibactin, along with colibactin 788, 406, 416, 420, and 430 derived from colibactin 770 through structural rearrangements and solvolysis. Furthermore, we were able to trap the degrading mature colibactin species by converting the diketone moiety into quinoxaline *in situ* in the crude culture extract to form colibactin 860 at milligram scale. This allowed us to determine the stereochemically complex structure of the rearranged form of an intact colibactin, colibactin 788, in detail. Furthermore, our study suggested that we were capturing only a few percent of the actual colibactin produced by the microbe, providing a crude quantitative insight into the inherent instability of this compound. Through the structural assignment of colibactins and their degradative products by the combination of LC-HRMS and NMR spectroscopies, we were able to elucidate further the fate of inherently unstable colibactin, which could help acquire a more complete picture of colibactin metabolism and identify key DNA adducts and biomarkers for diagnosing colorectal cancer.
Keywords: colibactin, risk factor for colorectal cancer, structural analysis

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田中理恵, 河村麻衣子, 袴塚高志, 花尻（木倉）瑠理: シート状危険ドラッグ製品中のLSD誘導体の同定と分析の検討.
薬学雑誌 2020;140:739-750. doi: 10.1248/yakushi.19-00230

To prevent the abuse of new psychoactive substances (NPS), a total of 2372 substances and two plants are controlled as "Designated Substances" in Japan as of September 2019. Although the distribution of these substances has decreased for the past three years, newly-emerged NPS are still being found. In this study, we detected four lysergic acid diethylamide (LSD) derivatives as designer drugs from four paper sheet products, which were obtained from 2014 to 2017 in Japan. The compounds were identified as 4-Acetyl-*N*,*N*-diethyl-7-methyl-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxamide (ALD-52), *N*,*N*,7-triethyl-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxamide (ETH-LAD), 7-Allyl-*N*,*N*-diethyl-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxamide (AL-LAD), *N*,*N*-diethyl-7-methyl-4-propionyl-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxamide (1P-LSD), by GC-MS, LC-MS, LC-Q-TOF-MS and NMR analyses. Further, we studied the extraction methods of LSD derivatives from paper sheet, and the analytical conditions of GC-MS, LC-MS and LC-FL(fluorescence). Among LSD derivatives, 1P-LSD have been controlled as designated substances (Shitei Yakubutsu) under the Pharmaceutical and Medical Device Act in Japan since April 2016. For the legislation of the other derivatives identified in this study, the evaluation of their pharmacological properties are now in progress.
Keywords: lysergic acid diethylamide derivatives, lysergamides, blotter paper, new psychoactive

substances

田中理恵，河村麻衣子，袴塚高志，花尻（木倉）瑠理：シート状危険ドラッグ製品中のLSD誘導体1cP-LSD，MIPLA，1B-LSDの同定．
薬学雑誌 2020;140:1405-1413

To prevent the abuse of new psychoactive substances (NPSs), a total of 2385 substances and two plants are controlled as "Designated Substances" in Japan as of Aril 2020. Although the distribution of these substances has decreased for the past five years, newly-emerged NPS are still being found. Recently, lysergic acid diethylamide (LSD) derivatives have appeared as designer drugs, all over the world. In this study, we detected three LSD derivatives from three paper sheet products, which were obtained from September 2019 to March 2020 in Japan. The compounds were identified as 4-cyclopropionyl-*N*,*N*-diethyl-7-methyl-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxamide (1cP-LSD), *N*-methyl-*N*-isopropyl-7-methyl-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxamide (MIPLA), 4-butyryl-*N*,*N*-diethyl-7-methyl-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxamide (1B-LSD), by GC-MS, LC-MS, LC-Q-TOF-MS and NMR analyses.
Keywords: lysergic acid diethylamide derivative, lysergamide, blotter paper, new psychoactive substance

Tanaka R, Kawamura M, Hakamatsuka T, Kikura-Hanajiri R: Identification of six tryptamine derivatives as designer drugs in illegal products.
Forensic Toxicology 2021;39:248-258

Purpose To prevent the abuse of new designer drugs, Japan has declared 2385 substances and two plants as "Designated Substances" as of March 2020. Although the distribution of these substances have decreased over the past five years, newly detected designer drugs are still being found. We have detected six designer drugs in six powdery products between February of 2017 and April of 2019. Methods The structures of the compounds were determined by gas chromatography mass spectrometry (GC-MS), liquid chromatography mass spectrometry (LC-MS), liquid chromatography with hybrid quadrupole time-of-flight mass spectrometry (LC-Q-TOF-MS) and nuclear magnetic resonance (NMR).

Results Six tryptamine derivatives (4-acetoxy-*N*,*N*-dipropyltryptamine, 4-hydroxy-*N*,*N*-dipropyltryptamine, 4-hydroxy-*N*-methyl-*N*-propyltryptamine, *N*-ethyl-*N*-propyltryptamine, 4-hydroxy-*N*-ethyl-*N*-propyltryptamine (4OH-EPT), and 4-hydroxy-*N*-methyl-*N*-cyclopropyltryptamine (4OH-McPT) were identified Among these, 4OH-EPT and 4OH-McPT were identified as newly distributed designer drugs.

Conclusions The continuous provisional monitoring of newly detected compounds in illicit products will largely prevent the distribution of these products.
Keywords: tryptamine derivative, illicit product, new psychoactive substances

Morita I*, Oyama H*, Kiguchi Y*, Ohuri A*, Fujimoto N*, Takeuchi A*, Tanaka R, Ogata J, Kikura-Hanajiri R, Kobayashi N*: Immunochemical monitoring of psilocybin and psilocin to identify hallucinogenic mushrooms.
J. Pharmaceut. Biomed. Anal. 2020;190:113485. doi: 10.1016/j.jpba.2020.113485.

Development of rapid and reliable immunochemical methods for monitoring psilocybin (4-phosphoryloxy-*N*,*N*-dimethyltryptamine; Pyb) and psilocin (dephosphorylated metabolite; Psi), the psychoactive compounds contained within hallucinogenic mushrooms (magic mushrooms), is desitable in order to identify these mushrooms and regulate their illicit use. Because no antibody was publicly avail-able for this purpose, we generated two independent monoclonal antibodies (mAbs) against Pyb or Psi, and then developed enzyme-linked immunosorbent assays (ELISAs) by using them. To generate the specific antibodies, novel immunogenic conjugates were prepared by linking Pyb or Psi molecules to carrier proteins by modifying their 2-(*N*,*N*-dimethylamino) ethyl side chains. Spleen cells from mice immunized with these conjugates were fused with P3/NS1/1-Ag4-1 myeloma cells, and hybridoma clones secreting anti-Pyb and anti-Psi mAbs were established. These mAbs were characterized for their biochemical features and then applied to competitive ELISAs, which used microplates coated with Pyb or Psi linked with albumin. These ELISAs enabled the determination of Pyb or Psi with measurable ranges of ca. 0.20-20 or 0.040-2.0 μg/assay (limit of detection was 0.14 or 0.029 μg/assay), respectively. The related tryptamines

were satisfactorily discriminated as exemplified by the cross-reactivity of the ELISA to determine Pyb (or Psi) with Psi (or Pyb) that were found to be 2.8% (or＜0.5%), respectively. The Pyb and Psi contents in a dried powder of the hallucinogenic mushroom, *Psilocybe cubensis*, were determined to be 0.39 and 0.32 (w/w)%, respectively. The ELISAs developed using the current mAbs are promising tools for identifying illegal hallucinogenic mushrooms.

Keywords: Psilocybin, Psilocin, Hallucinogenic mushroom

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緒方潤，河村麻衣子，袴塚高志，花尻（木倉）瑠理：改良PCR-RFLP法によるKratom製品の識別.
薬学雑誌 2020;140:1501-1508. doi:10.1248/yakushi.20-00170

In this study, the origins of 16 Kratom products obtained from the illegal drug market in Japan were investigated by DNA analyses and LC-MS analyses. When the PCR-restriction fragment length polymorphism (RFLP) was performed using the restriction enzyme XmaI, the same DNA fragment patterns were obtained from all 16 products. On the other hand, as a result of the identification of the plant species of each product by nucleotide sequence analyses, the sequences of *M. speciosa* were detected in only 14 products. Moreover, the DNA amplicons of the other psychotropic plant (*Mesembryanthemum* sp., "Kanna") were detected. This plant PCR amplicon has the restriction site for the XmaI at the same position of the *M. speciosa* PCR amplicon and it is difficult to distinguish "Kratom" and "Kanna" by the conventional PCR-RFLP. When the restriction enzyme XhoI was used simultaneously with the XmaI, the specific DNA fragment was only observed from the *M. speciosa* amplicon and it was possible to distinguish both species using this improved PCR-RFLP method.

Keywords: *Mitragyna speciosa*, PCR-restriction fragment length polymorphism (RFLP), *Mesembryanthemum*

Watanabe T[*1,2], Yasuda S, Kusakawa S, Kuroda T, Futamura M[*2,3], Ogawa M[*2,4], Mochizuki H[*2,5], Kikkawa E[*2,6], Furukawa H[*2,7], Nagaoka M[*2,8], Sato Y: Multisite studies for validation and improvement of a highly efficient culture assay for detection of undifferentiated human pluripotent stem cells intermingled in cell therapy products.
Cytotherapy. 2021;23:176-83. doi: 10.1016/j.jcyt.2020.07.009

Background aims: The Multisite Evaluation Study on Analytical Methods for Non-Clinical Safety Assessment of Human-Derived Regenerative Medical Products (MEASURE) is a Japanese experimental public-private partnership initiative, which aims to standardize methodology for tumorigenicity evaluation of human pluripotent stem cell (hPSC)-derived cell therapy products (CTPs). Undifferentiated hPSCs possess tumorigenic potential, and thus residual undifferentiated hPSCs are one of the major hazards for the risk of tumor formation from hPSC-derived CTPs. Among currently available assays, a highly efficient culture (HEC) assay is reported to be one of the most sensitive for the detection of residual undifferentiated hPSCs. *Methods:* MEASURE first validated the detection sensitivity of HEC assay and then investigated the feasibility of magnetic-activated cell sorting (MACS) to improve sensitivity. *Results:* The multisite experiments confirmed that the lower limit of detection under various conditions to which the human induced pluripotent stem cell lines and culture medium/substrate were subjected was 0.001%. In addition, MACS concentrated cells expressing undifferentiated cell markers and consequently achieved a detection sensitivity of 0.00002%. *Conclusions:* These results indicate that HEC assay is highly sensitive and robust and that the application of MACS on this assay is a promising tool for further mitigation of the potential tumorigenicity risk of hPSC-derived CTPs.

Keywords: tumorigenicity, multisite experiment, pluripotent stem cell

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Sato Y: A highly sensitive method for the detection of recombinant PERV-A/C env RNA using next generation sequencing technologies.
Sci. Rep., 2020;10:21935. doi: 10.1038/s41598-020-78890-2

Several xenogenic cell-based therapeutic products are currently under development around the world for the treatment of human diseases. Porcine islet cell products for treating human diabetes are a typical example. Since porcine cells possess endogenous retrovirus (PERV), which can replicate in human cells in vitro, the potential transmission of PERV has raised concerns in the development of these products. Four subgroups of infectious PERV have been identified, namely PERV-A, -B, -C, and recombinant PERV-A/C. Among them, PERV-A/C shows a high titre and there was a paper reported that an incidence of PERV-A/C viremia was increased in diseased pigs; thus, it would be important to monitor the emergence of PERV-A/C after transplantation of porcine products. In this study, we developed a highly sensitive method for the detection of PERV-A/C using next generation sequencing (NGS) technologies. A model PERV-C spiked with various doses of PERV-A/C were amplified by RT-PCR and the amplicons were analysed by NGS. We found that the NGS analysis allowed the detection of PERV-A/C at the abundance ratios of 1% and 0.1% with true positive rates of 100% and 57%, respectively, indicating that it would be useful for the rapid detection of PERV-A/C emergence after transplantation of porcine products.
Keywords: xenogenic cell-based therapeutic product, porcine endogenous retrovirus, next generation sequencing technology

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Miura T, Yasuda S, Sato Y: A simple method to estimate the in-house limit of detection for genetic mutations with low allele frequencies in whole-exome sequencing analysis by next-generation sequencing.
BMC Genomic Data, 2021;22:8. doi: 10.1186/s12863-020-00956-x

Background: Next-generation sequencing (NGS) has profoundly changed the approach to genetic/genomic research. Particularly, the clinical utility of NGS in detecting mutations associated with disease risk has contributed to the development of effective therapeutic strategies. Recently, comprehensive analysis of somatic genetic mutations by NGS has also been used as a new approach for controlling the quality of cell substrates for manufacturing biopharmaceuticals. However, the quality evaluation of cell substrates by NGS largely depends on the limit of detection (LOD) for rare somatic mutations. The purpose of this study was to develop a simple method for evaluating the ability of whole-exome sequencing (WES) by NGS to detect mutations with low allele frequency. To estimate the LOD of WES for low-frequency somatic mutations, we repeatedly and independently performed WES of a reference genomic DNA using the same NGS platform and assay design. LOD was defined as the allele frequency with a relative standard deviation (RSD) value of 30% and was estimated by a moving average curve of the relation between RSD and allele frequency. Results: Allele frequencies of 20 mutations in the reference material that had been pre-validated by droplet digital PCR (ddPCR) were obtained from 5, 15, 30, or 40 G base pair (Gbp) sequencing data per run. There was a significant association between the allele frequencies measured by WES and those pre-validated by ddPCR, whose p-value decreased as the sequencing data size increased. By this method, the LOD of allele frequency in WES with the sequencing data of 15 Gbp or more was estimated to be between 5 and 10%. Conclusions: For properly interpreting the WES data of somatic genetic mutations, it is necessary to have a cutoff threshold of low allele frequencies. The in-house LOD estimated by the simple method shown in this study provides a rationale for setting the cutoff.
Keywords: limit of detection, next-generation sequencing, allele frequency

澤田留美，佐藤陽治：再生医療等製品（細胞加工製品）の原料等としてのMSC（間葉系幹細胞／間葉系間質細胞）の品質特性解析の問題点―開発企業へのアンケート調査から見えてきたこと―.
再生医療 2020;19:52-64.

国内のMSC（間葉系幹細胞／間葉系間質細胞）加工製品開発企業の開発者から意見を聴収し取り纏め，再生医療等製品（細胞加工製品）の原料等としてのMSCの品質特性解析について開発者が考える問題点・課題を明らかした.
Keywords：間葉系幹細胞／間葉系間質細胞，再生医療

等製品，細胞加工製品

You X*, Suresh T, Liu W*, Cao Y*, Naito M, Furihata C, Honma M, Luan Y*, Suzuki T: Detection of genome-wide low-frequency mutations with Paired-End and Complementary Consensus Sequencing (PECC-Seq) revealed end-repair-derived artifacts as residual errors.

Arch Toxicol. 2020;94:3475-85. doi: 10.1007/s00204-020-02832-0

To improve the accuracy and the cost-efficiency of next-generation sequencing in ultralow-frequency mutation detection, we developed the Paired-End and Complementary Consensus Sequencing (PECC-Seq), a PCR-free duplex consensus sequencing approach. With the high accuracy of PECC-Seq, we identified the characteristic base substitution errors introduced by the end-repair process of mechanical fragmentation-based library preparations, which were prominent at the terminal 7 bp of the library fragments in the 5'-NpCpA-3' and 5'-NpCpT-3' trinucleotide context. As demonstrated at the human genome scale (TK6 cells), after removing these potential end-repair artifacts from the terminal 7 bp, PECC-Seq could reduce the sequencing error frequency to mid-10-7 with a relatively low sequencing depth. In mutagen-treated (6 μg/mL methyl methanesulfonate or 12 μg/mL N-nitroso-N-ethylurea) TK6, increases in mutagen treatment-related mutant frequencies could be detected, indicating the potential of PECC-Seq in detecting genome-wide ultra-rare mutations. In addition, our finding on the patterns of end-repair artifacts may provide new insights into further reducing technical errors not only for PECC-Seq, but also for other next-generation sequencing techniques.

Keywords: NGS, mutation, end-repair

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Kitamura Y*1, Suzuki T, Kohara A*2, Saeki KI*1: Hepatocarcinogen 4-methylquinoline induced G:C to C:G transversions in the *cII* gene in the liver of lambda/lacZ transgenic mice (Muta™Mouse).

Mutat Res. 2020;821:111709. doi: 10.1016/j.mrfmmm.2020.111709

We examined the effect of 4-MeQ on mutagenesis in the lambda *cII* gene in the liver of the Muta™Mouse, and we analyzed the sequences of the mutated genes. The mutation frequency of the liver *cII* gene was seven times higher in 4-MeQ-treated mice than in control mice. Sequence analysis revealed that 4-MeQ primarily induced G:C to C:G transversions (37 of 45). The specificities of 4-MeQ for target organ and mutation pattern were very consistent with those of quinoline. Thus, we showed that 4-MeQ was also genotoxic in the liver of the Muta™Mouse, and as with quinoline, the G:C to C:G transversion was the molecular signature of the 4-MeQ-induced mutations.

Keywords: 4-Methylquinoline, mutation spectrum, Muta™Mouse

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Nishikawa S*, Inoue Y*, Hori Y*, Miyajima C*, Morishita D*, Ohoka N, Hida S*, Makino T*, Hayashi H*: Anti-Inflammatory Activity of Kurarinone Involves Induction of HO-1 via the KEAP1/Nrf2 Pathway.

Antioxidants. 2020;9:842. doi: 10.3390/antiox9090842

Kurarinone, a flavonoid isolated from the roots of *Sophora flavescens*, was suggested to exert potent antioxidant and immunosuppressive effects. However, the underlying mechanisms remain unclear. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a key transcription factor that regulates the antioxidant defense system with anti-inflammatory activity. In the present study, we demonstrated that kurarinone activated Nrf2 and increased the expression of antioxidant enzymes, including heme oxygenase-1 (HO-1). Mechanistically, kurarinone downregulated the expression of kelch-like ECH-associated protein 1 (KEAP1), subsequently leading to the activation of Nrf2. Kurarinone also inhibited the expression of the inflammatory cytokine, interleukin (IL)-1β, and inducible nitric oxide synthase (iNos) in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages. The overexpression of HO-1 suppressed the LPS-induced production of inflammatory mediators in RAW264.7 cells, and the immunosuppressive effects of kurarinone were partially inhibited by a treatment with Tin Protomorphyrin IX (TinPPIX), an inhibitor of

HO-1. These results indicate that kurarinone activates the KEAP1/Nrf2 pathway to induce HO-1 expression, thereby exerting immunosuppressive effects.
Keywords: HO-1, KEAP1, *Sophora flavescens*

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Umemura K[*1], Ohtsuki S[*1], Nagaoka M[*2], Kusamori K[*2], Inoue T, Takahashi Y[*1], Takakura Y[*1], Nishikawa M[*1,2]: Critical contribution of macrophage scavenger receptor 1 to the uptake of nanostructured DNA by immune cells.
Nanomedicine. 2021;34:102386. doi: 10.1016/j.nano.2021.102386

Despite the efficient uptake of polypod-like nanostructured DNA, or polypodna, by macrophage-like RAW264.7 and other immune cells, the detailed mechanism has not been fully elucidated. Our previous study using HEK-Blue hTLR9 cells showed that transfection of macrophage scavenger receptor 1 (MSR1) increased the uptake of tetrapod-like structured DNA. Here, we investigated the involvement of MSR1 in the structure-dependent uptake of polypodna. Transfection of MSR1 to HEK-Blue hTLR9 cells pod number-dependently increased the uptake of polypodna, and its knockout in RAW264.7 cells reduced the uptake and subsequent cytokine release. To examine the binding of DNA with MSR1, biotinylated DNA added to RAW264.7 cells was cross-linked with cell surface proteins. Then, MSR1 cross-linked with polypodna, but not with single-stranded DNA. Similar results were obtained with murine primary immune cells. Taken together, MSR1 discriminates between simple and nanostructured DNAs and plays a dominant role in the efficient uptake of polypodna by immune cells.
Keywords: CRISPR/Cas9, DNA nanostructure, DNA uptake

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Kato R, Miyajima A, Komoriya K, Haishima Y: Novel cytokine marker available for skin irritation testing of medical devices using reconstructed human epidermis models.
Toxicol In Vitro, 2020:68 doi: 10.1016/j.tiv.2020.104919.

In biological safety evaluation of medical devices, false-negative results have been observed during skin irritation testing using the reconstructed human epidermis (RhE) model when measuring cell viability as a single marker. Therefore, to improve testing accuracy, this study conducted a comprehensive survey and performance evaluation of cytokines to identify a second marker. In addition to IL-1α, macrophage migration inhibitory factor (MIF) was newly identified as a candidate marker, in the Bio-Plex assay of EpiDerm model exposed to polymer sample extracts. Irritation based on cell viability level was not accurately determined in LabCyte model using silicone spiked with 25% heptanoic acid (HA). By contrast, the irritation potency was accurately assessed in detail by measuring IL-1α or MIF. Further, IL-1α and MIF levels in EpiDerm, LabCyte, and EpiSkin models stimulated with sodium dodecyl sulfate (SDS) were inversely correlated with cell viability, and were detected even at low SDS concentrations without cell toxicity. Additionally, MIF demonstrated greater S/N ratio and dose-dependency at high SDS concentrations in some models compared to IL-1α. These results indicated that MIF might be a useful second marker for improving the sensitivity and accuracy of skin irritation testing with RhE models.
Keywords: skin irritation, reconstructed human epidermis, macrophage migration inhibitory factors

Miyajima A, Kuroda Y, Sakemi-Hoshikawa K, Usami M[*1], Mitsunaga K[*2], Irie T, Ohno Y[*3], Sunouchi M: Inhibitory and inductive effects of 4- or 5-methyl-2-mercaptobenzimidazole, thyrotoxic and hepatotoxic rubber antioxidants, on several forms of cytochrome P450 in primary cultured rat and human hepatocytes.
Toxicol. Rep. 2020:7:979 doi: 10.1016/j.toxrep.2020.08.003.

Effects of 4-methyl-2-mercaptobenzimidazole (4-MeMBI) and 5-methyl-2- mercaptobenzimidazole (5-MeMBI) on cytochrome P450 (CYP) activity were examined in primary cultured rat hepatocytes. Hepatocytes from male Wistar rats were cultured in the presence of 4-MeMBI or 5-MeMBI (0-400 μM), and

the activity of CYPs 3A2/4 (48 and 96 h) and 1A1/2 (48 h) was determined by measuring the activity of testosterone 6β-hydroxylation and 7-ethoxyresorufin O-deethylation, respectively. As a result, 4-MeMBI and 5-MeMBI (≥12.5 μM) inhibited CYP3A2 activity. On the other hand, 4-MeMBI (≥25 μM) and 5-MeMBI (≥100 μM) induced CYP1A1/2 activity, being consistent with the previous in vivo results. In a comparative metabolism study using primary cultured human hepatocytes from two Caucasian donors, 4-MeMBI and 5-MeMBI induced the activity of CYPs 3A4 and 1A1/2 with individual variability. It was concluded from these results that 4-MeMBI, 5-MeMBI and MBI caused inhibition of CYP3A2 activity in primary cultured rat hepatocytes, suggesting their potential for metabolic drug-drug interactions. Primary cultured rat and human hepatocytes were considered to be useful for the evaluation of effects of the benzimidazole compounds on their inducibility and inhibitory activities of cytochrome P450 forms.

Keywords: benzimidazole, cytochrome P450, drug-metabolizing activity, hepatocyte, primary culture

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Fukunaga J†*1, Nomura Y†, Tanaka Y*1,2, Torigoe H*3, Nakamura Y*4,5, Sakamoto T*6, Kozu T*1 : A G-quadruplex-forming RNA aptamer binds to the MTG8 TAFH domain and dissociates the leukemic AML1-MTG8 fusion protein from DNA.

FEBS Lett. 2020;594:3477-3489, doi: 10.1002/1873-3468.13914

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MTG8 (RUNX1T1) is a fusion partner of AML1 (RUNX1) in the leukemic chromosome translocation t(8;21). The AML1-MTG8 fusion gene encodes a chimeric transcription factor. One of the highly conserved domains of MTG8 is TAFH which possesses homology with human TAF4 [TATA-box binding protein-associated factor]. To obtain specific inhibitors of the AML1-MTG8 fusion protein, we isolated RNA aptamers against the MTG8 TAFH domain using systematic evolution of ligands by exponential enrichment. All TAF aptamers contained guanine-rich sequences. Analyses of a TAF aptamer by NMR, CD, and mutagenesis revealed that it forms a parallel G-quadruplex structure in the presence of K+. Furthermore, the aptamer could bind to the AML1-MTG8 fusion protein and dissociate the AML1-MTG8/DNA complex, suggesting that it can inhibit the dominant negative effects of AML1-MTG8 against normal AML1 function and serve as a potential therapeutic agent for leukemia.

Keywords: RNA aptamer, AML1-MTG8 fusion protein, NMR

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Nomura Y, Yamamura J*1, Fukui C, Fujimaki H*2, Sakamoto K*1, Matsuo K*1, Kuromatsu H*1, Kikuchi Y, Haishima Y: Performance evaluation of bactericidal effect and endotoxin inactivation by low-temperature ozone/hydrogen peroxide mixed gas exposure.

J. Biomed. Mater. Res. Part B. 2021;Mar 29. doi: 10.1002/jbm.b.34840

This study evaluated the performance of a new O_3/H_2O_2 mixed gas sterilization instrument for killing microorganisms and inactivating bacterial endotoxin at low temperatures. Sterility assurance level was achieved by an over 6-log reduction of *Geobacillus stearothermophilus* ATCC 12980, and the decimal reduction value was 0.77 min in sterilization mode. A reduction of over 3 logs in Limulus amebocyte lysate coagulation activity of purified endotoxin from *Escherichia coli* was observed after treatment in endotoxin-inactivation mode. The same inactivation ability was observed when treating dried bacterial cells. Biomaterials made of polymer or metal did not exhibit cytotoxicity after gas exposure at O_3 concentrations below 200 ppm. As the results of human cell-based pyrogen testing, significant amounts of endotoxin that were over the limit for medical devices contacting cerebrospinal fluid (2.15 EU/device) were detected on scissors washed with a washer-disinfector and sterilized with ethylene oxide or autoclaving. In

contrast, endotoxin decreased to 0.29 ± 0.05 EU/device after O_3/H_2O_2 mixed gas sterilization in endotoxin-inactivation mode. Compared to conventional gas sterilization methods, O_3/H_2O_2 mixed gas has high sterilization ability and a strong capacity to inactivate endotoxin. It is expected that this sterilization technology will improve the safety of reusable medical devices and utensils for regenerative medicine.

Keywords: gas sterilization, endotoxin inactivation, reusable medical device

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Sakoda H, Okamoto Y, Haishima Y: In vitro estimation of reduction in strength and wear resistance of UHMWPE for joint prostheses due to lipid-induced degradation.
Journal of Biomedical Materials Research. Part B. Applied Biomaterials 2020;108B:3155-3161. doi:10.1002/jbm.b.34641

Ultra-high molecular weight polyethylene (UHMWPE) is used as a bearing surface of joint prostheses and has been reported to absorb lipids such as squalene (SQ) and cholesterol esters in vivo. These lipids have been suggested by in vitro studies using SQ as a model lipid to have the potential to induce polymer degradation. However, the impact of lipid-induced degradation on the strength and wear resistance of UHMWPE is unknown. In this study, lipid-induced degradation was simulated by SQ absorption and subsequent accelerated aging, and its influence on the strength and wear resistance of UHMWPE was investigated using wear, fatigue crack growth, and delamination testing. Lipid-induced degradation was found to have little impact on fatigue crack growth rates and delamination resistance. These results were consistent with previous reports that lipid-induced degradation is localized near the surface. However, we also found that lipid-induced degradation increased the wear rate of both non-crosslinked and crosslinked UHMWPE by a factor of 2.5 and 14, respectively. These results indicate that lipid-induced degradation may affect the durability and long-term clinical outcome of joint replacements due to increased wear of UHMWPE.

Keywords: delamination, fatigue, squalene, ultra-high molecular weight polyethylene, wear

Sakoda H, Sugano N*, Okamoto Y, Haishima Y: A novel method to eliminate the influence of absorbed lipids on the characterization of ultra-high molecular weight polyethylene using Fourier-transform infrared spectroscopy.
Bio-medical materials and engineering 2020;31(2):119-129. doi:10.3233/BME-201084

BACKGROUND: Fourier-transform infrared spectroscopy (FTIR) is one of the standard methods to analyze ultra-high molecular weight polyethylene (UHMWPE) in orthopedic implants. For retrieved components, lipid extraction using an organic solvent prior to the measurement is necessary to eliminate the influence of lipids absorbed in vivo. However, its influence on the measurement has not been substantially investigated.
OBJECTIVE: To investigate the influence of lipid extraction on the FTIR analysis of UHMWPE and to develop a novel method to obtain reliable results without inconvenient lipid extraction.
METHODS: FTIR analysis was repeatedly performed on UHMWPE specimens from retrieved components before and after lipid extraction under various conditions. A method to calculate the extent of influence of the absorbed lipids from the FTIR spectra was developed using a peak separation technique.
RESULTS: An elevated temperature was necessary for lipid extraction; however, it had the potential to influence the results if the conditions were not properly controlled. The results obtained using the peak separation technique coincided with those obtained after lipid extraction.
CONCLUSION: The use of the peak separation technique enables the efficient acquisition of reliable results without the need for lipid extraction.

Keywords: oxidation index, lipid index, retrieval study, oxidative degradation, lipid index subtracted oxidation index (s-OI)

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Tomita H*1, Kim SH*2, Inuzuka R*3, Matsui H*3, Tachimori H*4, Kobayashi T*5, Kato A*6, Fujii T*1, Haishima Y, Okamoto Y, Sakoda H: Stent

implantation for congenital heart diseases in Japan; Comprehensive analysis from the Japanese Society of Congenital Interventional Cardiology Registry.
Circulation Journal [Advance publication] Released: March 11, 2021. doi: 10.1253/circj.CJ-20-0915

Background: Stent implantation for vascular stenosis associated with congenital heart diseases is commonly performed as an off-label procedure in Japan because there is no officially approved stent for any congenital heart disease.
Methods and Results: We analyzed data from the Japanese Society of Congenital Interventional Cardiology Registry collected from January 2016 to December 2018. Patients who underwent stent implantation were enrolled in the present analysis. During the study period, there were 470 procedures, 443 sessions, and 391 cases. Of 443 sessions, 427 (96.4%) succeeded procedurally. There were no differences in the procedural success rates among age groups. In all, 416 sessions (367 patients; 94%) resulted in survival to 30 days after catheter intervention. Of 392 admissions, 357 patients (91%) survived to discharge. Only 4 deaths were directly related to stent implantation. Some in-hospital complications were observed during 55 of 443 sessions. Both hospital deaths and serious complications were significantly more frequent in the group with various preoperative risk factors.
Conclusions: Although not officially approved for congenital heart diseases in Japan, stent implantation in congenital heart diseases has been widely and routinely performed for many years with safety and efficacy. The aim of stenting was variable and broad because of many different applications and morphological variations. These data may facilitate approval of such an important device in Japan.
Keywords: catheter intervention, congenital heart disease, registry

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Tanino M†*, Uematsu M†, Nomura Y, Miyamoto Y, Haishima Y. Precautions for alkaline extraction of residual protein in cleanliness evaluation of reusable medical devices.
The Japanese Journal of Medical Instrumentation, 2020, 90(6):233-242.

† These authors contributed equally to this work.
We reassessed the residual protein quantitative method, combining 0.2 M NaOH extraction and the Coomassie brilliant blue (CBB) assay for the cleanliness evaluation of reusable medical devices. The bovine serum albumin (BSA) calibration curve slopes using various protein assay kits were significantly reduced under alkaline conditions, except with the Bradford reagent. When evaluating the characteristics of four CBB reagents, the Coomassie plus Protein Assay Kit and Protein Quantification Kit-Rapid directly quantified a small amount of BSA even under alkaline conditions. Variation in the BSA recovery rate was observed with Pierce™ 660 nm Protein Assay Reagent modified for micro assay and the Bradford reagent. In case of two assay kits other than CBB, contrary to the Sensolyte OPA Quantification Kit, the Micro BCA Assay Kit showed remarkable quantitativeness even under alkaline conditions. The recovery rate of the serum protein spiked on the stainless-steel plate with alkaline extraction was comparable to the extraction with water or mammalian protein extraction reagent. Polyacrylamide gel electrophoresis showed that BSA was significantly decomposed to peptide fragments by hydrolysis after alkaline treatments. These results indicated that residual protein extraction with 0.2 M NaOH is not essential, and accurate quantification depends upon the proper selection of a protein assay kit and the pH and protein concentration of the test solution.
Keywords: reusable medical devices, cleanliness evaluation, residual protein, extraction, guideline

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Zhang M*1, Tupin S*1, Anzai H*1, Kohata Y*1, Shojima M*2, Suzuki K*3, Okamoto Y, Tanaka K*4, Yagi T*5, Fujimura S*6,7, Ohta M*1. Implementation of computer simulation to assess flow diversion treatment outcomes: systematic review and meta-analysis.
J Neurointerv Surg. 2021 Feb;13(2):164-170. doi: 10.1136/neurintsurg-2020-016724.

Introduction: Despite a decade of research into virtual stent deployment and the post-stenting aneurysmal hemodynamics, the hemodynamic factors which correlate with successful treatment remain inconclusive. We aimed to examine the differences in various post-treatment hemodynamic parameters between successfully and unsuccessfully treated cases, and to quantify the additional flow diversion achievable through stent compaction or insertion of a second stent.

Methods: A systematic review and meta-analysis were performed on eligible studies published from 2000 to 2019. We first classified cases according to treatment success (aneurysm occlusion) and then calculated the pooled standardized mean differences (SMD) of each available parameter to examine their association with clinical outcomes. Any additional flow diversion arising from the two common strategies for improving the stent wire density was quantified by pooling the results of such studies.

Results: We found that differences in the aneurysmal inflow rate (SMD −6.05, 95% CI −10.87 to −1.23, p=0.01) and energy loss (SMD −5.28, 95% CI −7.09 to −3.46, p<0.001) between the successfully and unsuccessfully treated groups were indicative of statistical significance, in contrast to wall shear stress (p=0.37), intra-aneurysmal average velocity (p=0.09), vortex core-line length (p=0.46), and shear rate (p=0.09). Compacting a single stent could achieve additional flow diversion comparable to that by dual-stent implantation.

Conclusions: Inflow rate and energy loss have shown promise as identifiers to discriminate between successful and unsuccessful treatment, pending future research into their diagnostic performance to establish optimal cut-off values.

Keywords: aneurysm, blood flow, flow diverter

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Shojima M*1, Okamoto Y, Niizuma K*2, Ohta M*2, Ishikawa O*3, Fujisawa A*3, Tsukihara H*3, Sakai N*4, Tominaga T*2; Preliminary study of eye tracking to investigate the differences in gaze behaviors depending on the experience of neuroendovascular therapy,

Surg Neurol Int. 2020 Oct 21:11:351. doi: 10.25259/SNI_543_2020. eCollection 2020.

Background: Neuroendovascular therapy is now the choice for the management of many neurovascular pathologies, and physicians with endovascular skills are in high demand. In addition to the traditional method of practicing hand movements to learn skills, a new strategy of practicing eye movements to learn skills is also attracting attention. This preliminary study explored the differences in gaze behavior depending on experience with endovascular procedures to be facilitated in future skill training in neuroendovascular therapy.

Methods: Four physicians with experience of 3-412 neuroendovascular procedures wore eye-tracking devices during coil embolization of swine cervical arteries. Gaze metrics with direct correlations to the expertise of endovascular procedures were explored.

Results: Gaze metrics with a positive direct correlation to experience included the proportion of fixation durations (PFD) in the screen area and the native images. Those with a negative direct correlation included the PFD in the off-screen area and the roadmap images and the average fixation durations in the off-screen and coil areas. During the parent artery occlusion procedure with detachable coils, more experienced operators preferred to look at native images rather than roadmap images and that less experienced operators tended to look down at their hands more frequently.

Conclusion: This preliminary study demonstrated the feasibility of eye tracking to identify the differences in gaze behavior depending on the experience of endovascular procedures and may guide future eye-tracking studies in neuroendovascular therapy.

Keywords: coil embolization, experience, eye tracking, gaze behavior, skill learning

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Shojima M[*1], Okamoto Y, Ohta M[*2], Niizuma K[*2], Sakai N[*3], Tominaga T[*2]; Preliminary Study of Eye-Tracking During the Coil Insertion Task in a Silastic Model of Intracranial Aneurysms.

World Neurosurg. 2020 Jul; 139: e827-e835. doi: 10.1016/j.wneu.2020.05.012.

Objective: Surgical skills are generally acquired by watching the "hand movements" of experts. "Eye movements" are now attracting attention in skill-learning fields. Eye-tracking technology was introduced preliminarily to develop a better skill-learning system for neuroendovascular treatments.

Methods: During a task to place a detachable coil into a silastic cerebral aneurysm model under biplane X-ray fluoroscopy, gaze points were recorded using a head-mount eye-tracking device.

Results: During the task, 91% of fixations were allocated to the monitor displaying fluoroscopic images, and the others to the hands of operators or unspecified visual targets. More than 80% of fixations were located in frontal or lateral fluoroscopic images. Fixations were placed more frequently around the aneurysm than the microcatheter. One operator failed to recognize the timing when the proximal marker of the coil overlapped that of the microcatheter. The subject allocated most fixations to the frontal fluoroscopic image, whereas other subjects placed most fixations to the lateral fluoroscopic image. Furthermore, that operator put no fixations to the proximal marker of the microcatheter.

Conclusions: The results of this preliminary study imply the feasibility of the eye tracking-based learning system for neuroendovascular treatments. The eye-tracking analysis has potential in investigating or preventing procedural failures in neuroendovascular treatments.

Keywords: coil embolization, endovascular, eye-tracking, fixation, intracranial aneurysm, neurointervention, skill learning

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Katsumi E[*1], Oshima N, Kagawa N[*2], Ohara H [*2], Hada N[*1]: Changes in the extracted amounts and seasonally variable constituents of *Diospyros kaki* at different growth stage.

J Nat Med 2021;75:105-15. doi: 10.1007/s11418-020-01456-z

Persimmon Calyx is a crude drug derived from the persistent calyx of mature fruit of *Diospyros kaki* Thunberg (Ebenaceae) and is used for the treatment of intractable hiccups. Although there are several reports on the isolation of constituents from Persimmon Calyx, its active constituents have not been elucidated. In this study, by focusing on the medicinal part of Persimmon Calyx, calyx on mature fruit of *D. kaki*, we examined the changes in the extraction amounts of 3 cultivars of *D. kaki* ('Hiratanenashi', 'Jiro', and 'Tonewase') to identify and quantify seasonally variable constituents during the maturation process by analysing their chemical compositions. We found that the extraction weight of the calyx, fruit of persimmons, and total tannin content in calyxes were significantly increased during maturation. Lupeol (**1**), betulinic acid (**2**), pomolic acid (**3**), ursolic acid (**4**), β-sitosterol (**5**), rotungenic acid (**6**), barbinervic acid (**7**), catechin (**8**), gallocatechin (**9**), and sucrose (**10**) were identified in the calyx of *D. kaki*. Compounds **1**, **6**, and **7** were isolated from Persimmon Calyx for the first time. Moreover, the isolated compounds (**1**–**7**) and their analogue (oleanolic acid) were quantitatively analysed, and the results showed that the amounts of **4** and oleanolic acid were reduced during maturation, whereas that of **2**, **3**, **6**, and **7** were increased.

Keywords: Persimmon Calyx, *Diospyros kaki*, Triterpenes, Tannins, Seasonally variable constituents

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久保田領志，秋山卓美，五十嵐良明：マイクロ波分解－誘導結合プラズマ質量分析法による化粧品中の微量金属不純物分析法の検討.

日本香粧品学会誌 2020;44(4):289-94.

Currently, international discussions to establish acceptable trace heavy metal levels in finished cosmetic products and their testing methods are ongoing. In Japan, the testing methods and acceptable levels of heavy metals in raw materials have already been specified with the Japanese Standards of Quasi-

drug Ingredients, but no specification regarding their testing methods and levels in finished cosmetic products has been published. In order to assess trace metal impurity in cosmetic products, an analytical method for determining 13 metals in finished cosmetic products by using microwave-assisted digestion and inductively coupled plasma-mass spectrometry was developed and validated. Because cosmetic products contain ingredients in various dosage forms, including various fats, oils, pigments, dyes, and minerals, microwave digestion conditions for the preparation of sample solution were mainly investigated using oil-based reference standard solution and certified reference material (CRM) (Lake sediment CRM, for trace elements analysis). In the recovery test using an oil-based reference standard solution, good recovery and repeatability were achieved under the developed acid digestion conditions using HNO_3; HNO_3 and H_2O_2; and HNO_3 and HF. However, in terms of recovery from lake sediment CRM, good recovery from the CRM was achieved, and high concentrations (the value closest to the true concentration) in the commercial cosmetic products for each metals were obtained only when using HNO_3 and HF for digestion. Therefore, HNO_3 and HF were used in the microwave digestion treatment of commercial 59 lipsticks, 23 lip glosses, and 20 lip liners. The metals detected at high concentrations and frequencies were Zn, Sr, and Sn for lipstick; Zn and Sn for lip gloss; and Mn, Zn, Sr, and Sn for lip liner. These metals were likely derived from zinc oxide, tin oxide, and manganese violet those were labeled ingredients on the product. Compared to the recommended acceptable levels of Pb, As, Cd, and Sb in cosmetic products by International Cooperation on Cosmetics Regulation (ICCR) and Health Canada, the concentration of Sb in some of the tested products exceeded the acceptable level.

Keywords: Cosmetic products, heavy metals, microwave digestion-ICP-MS

小林憲弘，土屋裕子，五十嵐良明：塩素処理による水道水中プロチオホスの分解とプロチオホスオキソンの生成挙動.

水道協会雑誌 2020;89（9):2-11.

有機リン系殺虫剤の一種であるプロチオホスと，そのオキソン体（プロチオホスオキソン）の水道水中での同時分析条件について検討し，分析方法の妥当性を評価した．さらに，プロチオホスの塩素処理実験を行い，プロチオホスオキソンの生成挙動と水中での安定性を評価した．塩素処理実験の結果，プロチオホスの消失に伴いプロチオホスオキソンの生成が確認され，消失したプロチオホスのほぼ全量がプロチオホスオキソンに変換されたものと考えられた．生成したプロチオホスオキソンは，水中で長時間安定的に残存していたことから，水道水質検査においてはプロチオホスよりもプロチオホスオキソンを主体として検査すべきと考えられる.

Keywords：塩素処理，水道水，農薬

小林憲弘，土屋裕子，高木総吉*，五十嵐良明：水道水中農薬のGC/MSスクリーニング分析法の開発と実試料への適用.

環境科学会誌 2020;33:136–57. doi: 10.11353/sesj.33.136

水道水あるいは水道原水中の農薬を既存の標準検査方法よりも迅速・簡便に検査するため，標準品を測定せずに，予めデータベースに登録された情報を基に定性・定量を行う，「ターゲットスクリーニング分析法」の水道水質検査への適用について検討した．172農薬を対象として，GC/MSによるターゲットスクリーニング分析用のデータベースを２台の装置（GCMS-QP2010 Plus, JMS-Q1050GC）でそれぞれ構築し，装置による違いを比較した．さらに，これらのデータベースを用いて，水道水・水道原水等75試料を測定し，標準検査方法で測定した結果と比較することで，標準検査方法との定性・定量結果の違いについて考察した．本研究の結果から，検出される農薬を広く検索する目的で用いる場合，スクリーニング分析法は有用と考えられた.

Keywords：水道水，農薬，GC/MS

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Sugaya N*, Takahashi M*, Sakurai K*, Tahara M, Kawakami T: Headspace GC/MS analysis of residual solvents in dietary supplements, cosmetics, and household products using ethyl lactate as a dissolution medium.

J AOAC Int 2020;103:407-12. doi: 10.5740/jaoacint.19-0260

The static headspace technique is one of the most popular techniques for residual solvent analysis and dimethyl sulfoxide (DMSO) and *N*,*N*-dimethylformamide (DMF) are widely use as the dissolution media. This study aims to establish ethyl lactate (EL), a solvent with low toxicity and less environmental impact, as an alternative dissolution

medium to DMSO and DMF for the static headspace analysis of toxic residual solvents in food, cosmetics, and similar complex organic matrices. Samples (a sample of dietary supplement and two samples each of cosmetics and household products) spiked with benzene, carbon tetrachloride, 1,2-dichloroethane, 1,1-dichloroethene, and 1,1,1-trichloroethane were dissolved in EL, DMSO, and DMF. Static headspace GC/MS and the standard addition method were used to detect and quantify the residual solvents. The dissolution and dispersion of these samples, especially the ones which were water-insoluble, were better than those in DMSO and DMF. The recoveries, except that of benzene in an aerosol spray, in EL ranged from 77 to 110%. The relative SDs in EL ranged from 2.5 to 11% and were better or equivalent to those in DMSO and DMF. EL was suitable as the dissolution medium for such samples, which may contain large amounts of organic solvents or various ingredients, in static headspace GC/MS analysis of residual solvents.

Keywords: Headspace GC/MS, residual solvent, ethyl lactate

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Omura R[*1], Sowa-Osako J[*1], Okazaki A[*1], Tateishi C[*1], Fukai K [*1,2], Kawakami T, Tahara M, Tsuruta D[*1]: A case of allergic contact dermatitis owing to abietic acid derivative in an over-the-counter hydrocolloid dressing.

Contact Dermatitis 2020;82:309-10. doi: 10.1111/cod.13461

A 13-year-old girl had skin laceration on the left lower leg. She applied an over-the-counter hydrocolloid dressing ("Dressing A"). Next day, she replaced "Dressing A" with another over-the-counter hydrocolloid dressing ("Dressing B"). Two days later, severe pruritus developed, making her remove "Dressing B". She visited a local dermatologist and was referred to us. Physical examination revealed a quadrangular infiltrative erythema with central laceration on the left lower leg. Thus, we performed a closed patch testing with "Dressing A", "Dressing B", and rosin. Positive reactions were noted with "Dressing A" on D2, D3, and D7, and with rosin on D7. However, all results with "Dressing B" were negative. In "Dressing A", dihydroabietic acid, dehydroabietic acid, and methyl dehydroabietic acid were detected by chemical analysis. However, in "Dressing B," no abietic acid-related compounds were noted. We diagnosed this case as allergic contact dermatitis owing to abietic acid derivative in the hydrocolloid dressing "Dressing A".

Keywords: rosin, hydrocolloid dressing, contact dermatitis

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Akimoto M[*1], Yamamoto Y[*1], Watanabe S[*2], Yamaga H[*2], Yoshida K[*3],Wakabayashi K[*3], Tahara Yu[*3], Horie N[*4], Fujimoto K[*4], Kusakari K[*5], Kamiya K[*5], Kojima K[*6], Kawakami T, Kojima H, Ono A[*7], Kasahara T[*1], Fujita M[*1]: The effect of dimethyl sulfoxide (DMSO) on the oxidation of cysteine-derivative nucleophilic reagent when using acetonitrile containing DMSO as an solvent in the Amino acid Derivative Reactivity Assay (ADRA).

J Appl Toxicol 2020;40:843-54. doi: 10.1002/jat.3948

The amino acid derivative reactivity assay (ADRA), which is an in chemico alternative to the use of animals in testing for skin sensitization potential, offers significant advantages over the direct peptide reactivity assay (DPRA) in that it utilizes nucleophilic reagents that are sensitive enough to be used with test chemical solutions prepared to concentrations of 1 mM, which is one-hundredth that of DPRA. ADRA testing of hydrophobic or other poorly soluble compounds requires that they be dissolved in a solvent consisting of dimethyl sulfoxide (DMSO) and acetonitrile. DMSO is known to promote dimerization by oxidizing thiols, which then form disulfide bonds. We investigated the extent to which DMSO oxidizes the cysteine-derived nucleophilic reagents used in both DPRA and ADRA and found that oxidation of both *N*-(2-(-naphthyl) acetyl)-L-cysteine (NAC) and cysteine peptide increases as the concentration of DMSO increases, thereby lowering the concentration of the nucleophilic reagent. We also found that use of a solvent consisting of 5% DMSO in acetonitrile consistently lowered NAC concentrations by about 0.4 μM relative to the use of solvents containing no DMSO. We also tested nine sensitizers and four nonsensitizers having different sensitization potencies to compare NAC depletion with and without 5% DMSO and found that reactivity

was about the same with either solvent. Based on the above, we conclude that the use of a solvent containing 5% DMSO has no effect on the accuracy of ADRA test results. We plan to review and propose revisions to OECD Test Guideline 442C based on the above investigation.

Keywords: ADRA (amino acid derivative reactivity assay), dimethyl sulfoxide, oxidation

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西以和貴*，佐藤学*，中野富美*，辻清美*，上村仁*，河上強志：繊維製品中のディルドリン及びDTTB分析法の開発.

薬学雑誌 2020;140:809-18. doi: 10.1248/yakushi.19-00262

Standard analytical methods for the detection of dieldrin and 4,6-dichloro-7-(2,4,5-trichlorophenoxy)-2-trifluoromethylbenzimidazole (DTTB) in textiles, which are regulated by Japanese law ("Act on the Control of Household Products Containing Harmful Substances"), have been in place for more than 30 years. In this study, we developed an improved analytical method, based on GC-MS, that uses safe reagents and can simultaneously detect dieldrin and DTTB analytes. In the standard (existing) analytical method, dimethyl sulfate, which is a potential carcinogen, is used to derivatize DTTB. In the developed method, phenyltrimethylammonium hydroxide, as an alternative reagent, was used to derivatize DTTB in good results. Dieldrin and the derivatized DTTBs gave highly linear calibration curves when analyzed by GC-MS. Moreover, we found that both analytes are adequately extracted from textiles by refluxing in hydrochloric acid and methanol. Furthermore, we established a purification method using the Bond Elut PRS column that effectively removed interfering substances in woolen products. Finally, we developed an improved analysis method by combining the above-mentioned techniques; the developed method exhibited a recovery rate of 94-104% and a relative standard deviation of less than 7% for both analytes. In addition, the limits of quantitation (dieldrin: 1.3 μg/g, DTTB: 0.72 μg/g) were sufficiently lower than the Japanese regulatory value of 30 μg/g.

Keywords: dieldrin, textile, phenyltrimethylammonium

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Kawakami T, Isama K *1, Jinno H*2: Transferability of phthalic acid ester plasticizers and other plasticizers to skin using model PVC sheets.

J Environ Sci Health Part A 2020;55:1163-72. doi: 10.1080/10934529.2020.1795503

The transferability of phthalic acid esters (PAEs) and other plasticizers, from model polyvinyl chloride (PVC) sheets to the skin of 11 subjects was assessed by measuring the amount of substance transferred using PVC sheets containing PAEs and alternative plasticizers of different types and contents. For all subjects, the transferred amount, from sheets containing 28 wt% PAE or from mixed sheets containing 14 wt% each of di (2-ethylhexyl) phthalate (DEHP) and other PAE, was greater than that from sheets containing 15 wt% each of PAE or alternative plasticizer only. A comparison of the transferability of five types of PAE showed that transfer tended to occur more readily as the n-octanol-water partition coefficient increased, suggesting that PAE hydrophobicity affected its transferability. The transferability of the alternative plasticizers di (2-ethylhexyl) terephthalate and 1,2-cyclohexane dicarboxylic acid diisononyl ester showed a similar trend; however, the transferred amount tended to be higher from model PVC sheets containing 28 wt% PAE or mixed with DEHP. The transferability of PAEs and alternative plasticizers was higher for certain subjects, suggesting individual differences in the transferability of chemicals to the subject's skin surface and is the presence of a group of people comparatively more susceptible to such transfer.

Keywords: phthalic acid esters, polyvinyl chloride, skin transferability

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Kawakami T, Isama K *1, Ikarashi Y, Jinno H*2: Evaluation of the sensitization potential of volatile organic compounds (VOCs) and semi-volatile organic compounds (SVOCs) using the direct peptide reactivity assay (DPRA)

J Toxicol Sci 2020;45:725-35. doi: 10.2131/jts.45.725

The purpose of this study was to evaluate the sensitization potential of 82 compounds classified as volatile and/or semi-volatile organic compounds using the direct peptide reactivity assay (DPRA), given that these chemical compounds have been detected frequently and at high concentrations in a national survey of Japanese indoor air pollution and other studies. The skin sensitization potential of 81 of these compounds was evaluable in our study; one compound co-eluted with cysteine peptide and was therefore not evaluable. Twenty-five of the evaluated compounds were classified as positive. Although all glycols and plasticizers detected frequently and at high concentrations in a national survey of Japanese indoor air pollution were negative, hexanal and nonanal, which are found in fragrances and building materials, tested positive. Monoethanolamine and 1,3-butanediol, which cause clinical contact dermatitis, and several compounds reported to have weak sensitization potential in animal studies, were classified as negative. Thus, it was considered that compounds with weak sensitization potential were evaluated as negative in the DPRA. Although the sensitization potential of the formaldehyde-releasing preservative bronopol has been attributed to the release of formaldehyde (a well-known contact allergen) by its degradation, its degradation products—bromonitromethane and 2-bromoethanol—were classified as positive, indicating that these degradation products also exhibit sensitization potential. The compounds that tested positive in this study should be comprehensively assessed through multiple toxicity and epidemiological studies.

Keywords: direct peptide reactivity assay (DPRA), volatile and semi-volatile compounds, sensitization potential

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河上強志，菅谷なえ子*1，田原麻衣子，大嶋智子*2，西以和貴*3，上村仁*3，塩田寛子*4，鈴木郁雄*4，田畑佳世*5，五十嵐良明：有害物質を含有する家庭用品の規制に関する法律（有害物質含有家庭用品規制法）におけるメタノール，トリクロロエチレン及びテトラクロロエチレン試験法改定に係る検討

薬学雑誌 2020;140:1485-94. doi: 10.1248/yakushi.20-00163

In Japan, the use of methanol, trichloroethylene, and tetrachloroethylene in aerosol household products is banned under the Act on the Control of Household Products Containing Harmful Substances. As the official analytical methods for testing for these substances have not been revised for over 35 years, several issues have been pointed out. Thus, we developed a new method to revise the official method in our previous study. In this study, validation of the proposed method for detecting the target substances was conducted using two aerosol-product samples (A and B), which contained methanol, trichloroethylene, and tetrachloroethylene. Sample A comprised regulated values of these compounds, while sample B comprised one-tenth of the regulated amounts. They also contained several volatile compounds that served as interfering substances. Subsequently, the samples were analyzed using head space/gas chromatography-mass spectrometry, and it was confirmed that the three target substances were separated from the other chemicals on chromatograms. Validation tests were conducted at seven laboratories to evaluate the proposed method using the prepared samples. In one laboratory, the recovery of trichloroethylene and tetrachloroethylene in sample B was slightly higher at 120%, while the recoveries obtained from the other tests were between 70% and 120%. Relative standard deviation at each laboratory was less than 10%. Furthermore, the relative standard deviations between the validation tests with respect to each chemical were less than 15%. Therefore, the method validated in this study was considered to be effective as a revised method for testing for methanol, trichloroethylene, and trichloroethylene in household aerosol products.

Keywords: Head-space GC/MS, household aerosol

products, validation

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岩渕千雅子*1，栗田昴幸*1，日野治子*1，関東裕美*2，岩本正照*3，河上強志，田原麻衣子，松永佳世子*4：FreeStyleリブレによるアレルギー性接触皮膚炎

日皮会誌 2020;130:2557-65. doi: 10.14924/dermatol.130.2557

皮下間質液中のグルコースを測定する持続血糖モニターのFreeStyleリブレは2017年から使用され始めた．本邦でのアレルギー性接触皮膚炎の報告は今のところは稀であるが，今回，接着部テープによるアレルギー性接触皮膚炎を発症した糖尿病患者３例を報告する．３例中２例で行った成分パッチテストからテープ部に含まれるアクリル酸イソボルニル（IBOA）が原因アレルゲンであることが判明した．当院では15例が使用し，ほか２例も紅斑や瘙痒が出現していた．今後もFreeStyleリブレの使用例の増加が予想され，IBOAは注意すべきアレルゲンとして重要である．

Keywords：持続血糖モニター，アクリル酸イソボルニル，接触皮膚炎

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西以和貴*，上村仁*，河上強志：ヘリウムガス不足に対応した繊維製品中防虫加工剤の分析法

神奈川衛研報 2020;50:15-20.

有害物質を含有する家庭用品の規制に関する法律（家庭用品規制法）において，繊維製品に防虫剤として用いられるディルドリン及び4,6-ジクロル-7-(2,4,5-トリクロルフェノキシ)-2-トリフルオルメチルベンズイミダゾール（DTTB）が規制対象となっている．これらの物質に対する試験法は家庭用品規制法施行規則で定められているが，ディルドリンは昭和53年，DTTBは昭和57年の規制導入当初から試験法が改正されていない．我々はこれまでの研究で，現行の試験法よりも効率性・安全性に優れた新試験法を開発した．この試験法はヘリウムガスを使用するガスクロマトグラフ/質量分析計（GC/MS）を用いるものであるが，昨今ヘリウムガス供給不足が問題となっていることから，その代替分析機器が利用可能かを検討する必要がある．そこで本研究では，ヘリウムガスを使用せずに分析可能な水素キャリアガスGC/MS及び高速液体クロマトグラフ／フォトダイオードアレイ検出器（HPLC/PDA）の利用可能か否かについて検討を行った．その結果，いずれも感度の面でヘリウムキャリアガスGC/MSに劣るものの，現行基準値である30 μg/gを下回る定量下限値が得られることがわかった．

Keywords：ヘリウムガス，GC/MS，HPLC/PDA

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小林麻紀*，酒井奈穂子*，大町勇貴*，森田有香*，根本了，大塚健治*：LC-MS/MSによる畜産物中のアシュラム分析法．

食品衛生学雑誌 2021;62(1):1-7. doi: https://doi.org/10.3358/shokueishi.62.1

An analytical method based on LC-MS/MS was developed for the determination of asulam in livestock products. Asulam in livestock products was extracted with acetone. The crude extracts were defatted by acetonitrile and *n*-hexane partitioning. Cleanup was carried out using a combination of ethylenediamine-*N*-propyl silylation silica gel (PSA) and octadecyl silylated silica gel (C_{18}) mini columns with acidic condition. The sample solution was subjected to LC-MS/MS using an external solvent calibration curve. The average recovery (n=5)of Asulam from four types of livestock products (bovine muscle, bovine fat, bovine liver and milk) spike at the maximum residue limits (MRLs) or at a uniform limit of 0.01 mg/kg was 92.7-98.7%, with a relative standard deviation of 3.1-11.6%. The limit of quantitation of the developed method was calculated to be 0.01 mg/kg.

Keywords: asulam, livestock products, LC-MS/MS

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坂井隆敏，菊地博之，縄田裕美，根本了，穐山浩：LC-MS/MSを用いた畜産物中フィプロニルおよびフィプロニルスルホンの分析法．

食品衛生学雑誌 2020;61(5):171-177. doi: https://doi.org/10.3358/shokueishi.61.171

畜産物中のフィプロニルおよびその主要代謝物であるフィプロニルスルホンについて，LC-MS/MSを用いた迅速かつ高感度な分析法を開発した．試料からn-ヘキサ

ンおよび無水硫酸ナトリウム存在下，酢酸酸性下アセトニトリルで抽出し，中性アルミナカートリッジカラムを用いて精製した．測定はLC-MS/MSを用い，ESIによるネガティブイオンモードで行った．開発した分析法を用い，畜産物６食品について添加回収試験（各食品n＝５）を実施したところ，基準値相当濃度を添加した場合の真度および併行精度はフィプロニルでそれぞれ95～115および0.8～4.1％，フィプロニルスルホンでそれぞれ94～101および0.9～5.1％であった．また，添加濃度0.001 mg/kgの場合の真度および併行精度も良好であった．確立した分析法の定量下限値はフィプロニルおよびフィプロニルスルホンともに0.001 mg/kgと推定された．本分析法は畜産物中の基準値の適合性の判定に有用な方法と示唆された．

Keywords：フィプロニル，フィプロニルスルホン，LC-MS/MS

Saito-Shida S, Nagata M*, Nemoto S, Akiyama H: Multi-residue determination of pesticides in green tea by gas chromatography-tandem mass spectrometry with atmospheric pressure chemical ionisation using nitrogen as the carrier gas.

Food Addit Contam Part A 2021;38(1):125-135. doi: 10.1080/19440049.2020.1846082

Helium is commonly used as a carrier gas in gas chromatography-tandem mass spectrometry (GC-MS/MS); however, there are growing concerns regarding its global shortage and the resulting limited supply and high cost. Using nitrogen as an alternative carrier gas in GC-MS/MS with the widely used electron ionisation (EI) technique leads to a significantly lower sensitivity; thus, in this study, we explored the use of atmospheric-pressure chemical ionisation (APCI) as the ionisation method and examined the applicability of GC-(APCI)MS/MS with nitrogen gas for the determination of pesticide residues. GC-(APCI)MS/MS using nitrogen provided slightly wider peaks, and poorer isomeric separation compared to those using helium under identical conditions; however, the peak intensities were comparable. GC-(APCI)MS/MS using nitrogen was validated for 166 pesticides in green tea at a spiking level of 0.01 mg/kg and was compared with the conventional GC-(EI)MS/MS using helium gas. Except dimethomorph and resmethrin, GC-(APCI)MS/MS showed satisfactory results that were comparable to those of GC-(EI)MS/MS for most compounds, with trueness in the range of 73%-95% and relative standard deviations of <11%. The sensitivity and selectivity of GC-(APCI)MS/MS with nitrogen were superior to those of GC-(EI)MS/MS with helium. Therefore, GC-(APCI)MS/MS using nitrogen as the carrier gas, which has minimal concerns related to availability, could be a promising alternative to the conventional GC-(EI)MS/MS technique that employs helium.

Keywords: pesticides, atmospheric-pressure chemical ionisation, GC-MS/MS

* Waters Corporation

Saito-Shida S, Nemoto S, Akiyama H: Multiresidue method for determining multiclass acidic pesticides in agricultural foods by liquid chromatography-tandem mass spectrometry.

Anal Methods 2021;13(7):894-902. doi: 10.1039/D0AY02101F

A reliable multiresidue method was developed for determining multiclass acidic pesticides in cereal grains, legumes, vegetables, and fruits. The target pesticides comprise 75 compounds, including phenoxy acid, sulfonylurea, imidazoline, and triazolopyrimidine herbicides, with acidic dissociation constant (pKa) values of 1.9-5.9. The method includes extraction with acidified acetonitrile, salting out, cleanup with octadecyl silica and primary secondary amine cartridges, and subsequent liquid chromatography-tandem mass spectrometry. The analytical performance of the developed method was validated for nine foods (i.e., brown rice, soybeans, peanuts, spinach, cabbage, eggplant, potatoes, apples, and oranges) at a concentration of 0.01 mg/kg. Because matrix effects were negligible for most pesticide and food combinations, solvent-based calibration curves were used for quantification purposes. Most of the target compounds exhibited satisfactory analytical performance with trueness values of 70-100% and relative standard deviations below 14%. The high selectivity of the developed method was evidenced by the absence of interfering peaks near those of the target analytes. With the exception of 1-naphthaleneacetic acid, for which linearity was observed at 2.5-100 ng/mL, linear calibration curves were constructed for the target compounds in the 1-100 ng/mL range, with coefficients of determination

exceeding 0.995. The limits of detection were 3 μg/kg or below in the examined matrices. The results demonstrate that the developed method is suitable for monitoring acidic pesticides in a variety of foods.
Keywords: acidic pesticides, foods, LC-MS/MS

Saito-Shida S, Nemoto S, Akiyama H: Quantitative and confirmatory analysis of pesticide residues in cereal grains and legumes by liquid chromatography-quadrupole-time-of-flight mass spectrometry.
Foods 2021;10(1):78. doi: 10.3390/foods10010078

For controlling pesticide residues in food and ensuring food safety, multiresidue methods that can monitor a wide range of pesticides in various types of foods are required for regulatory monitoring. In this study, to demonstrate the applicability of liquid chromatography-quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) for quantitative and confirmatory analysis of pesticide residues in cereal grains and legumes, the LC-QTOF-MS method using full-scan acquisition was validated for 151 pesticides in brown rice, soybeans, and peanuts at a spiked level of 0.01 mg/kg. With the exception of 5 out of 151 target pesticides, sufficiently high signal intensities were obtained at 0.005 μg/mL (corresponding to 0.01 mg/kg). Trueness was in the range 70-95%, with intra- and inter-day precisions below 16% and 24%, respectively, with the exception of 7 pesticides in brown rice, 10 pesticides in soybeans, and 9 pesticides in peanuts. No interfering peaks were observed near the retention times of the target pesticides. Furthermore, information on accurate fragment-ion masses obtained by a data-independent acquisition enabled unambiguous confirmation. The results suggest that the LC-QTOF-MS method is suitable for pesticide residues' analysis of cereal grains and legumes, and can be utilized for regulatory routine analysis.
Keywords: pesticides, multiresidue method, LC-QTOF-MS

志田（齊藤）静夏，根本了，穐山浩：野菜・果実中の残留農薬分析における試料調製方法及び試料量による分析値のばらつきへの影響.
日本食品化学学会誌 2020;27(3):135-140. doi: 10.18891/jjfcs.27.3_135

Reducing the analytical portion size during pesticide residue analysis can potentially reduce the amounts of organic solvents and reagents used, as well as the time required for analysis. However, if sample processing is not performed properly and the concentration distribution of the pesticide residues in the homogenized sample is not uniform, the analytical portion may not represent the original sample, thereby leading to large variations in analytical values and incorrect results. In this study, we compared variations in the analytical values of incurred residues in various vegetable and fruit portions of different size (2-20 g) in order to examine how portion size affects the analytical values obtained during pesticide-residue analysis. The results show that variations in the analytical values are relatively small (relative standard deviation <10%) for foods that can form homogeneous samples relatively easily when appropriate sample processing methods were employed, even when a 2-g analytical portion was used for analysis. In contrast, for foods such as grapes that are not easily homogenized, large variations in the analytical values were observed for analytical portions less than 5 g in size due to variations in the distributions of pesticide residues in the sample. Furthermore, to examine the effect of the sample processing method on sample homogeneity, variations in the analytical values of incurred residues in tomato were compared using three methods, namely sample processing at room temperature using a household food processor, laboratory-knife milling, and cryogenic milling. Sample processing using a household food processor was found to provide large variations in the analytical values (relative standard deviation >20%), even when a 20-g portion was used for analysis. The results show that, compared to the other two methods, sample processing at room temperature using a household food processor may not provide sufficiently homogeneous samples.
Keywords: pesticide, analytical portion size, sample processing

菊地博之，坂井隆敏，大倉知子，根本了，穐山浩：ヘリウムガス供給不足に対応した農産物中の残留農薬等の LC-MS/MS を用いる一斉試験法の適用検討.
日本食品化学学会誌 2020;27(3):184-189. doi: https://doi.org/10.18891/jjfcs.27.3_184

Simultaneous official methods using GC-MS/MS and LC-MS/MS for quantifying residual pesticides in agricultural products are widely used at quarantine

stations, inspection laboratories, and prefectural institutes in Japan. The worldwide helium shortage led to limited helium availability and higher costs in 2019. To ensure the safety of foods, it is important to maintain a continuous monitoring system. We selected 31 pesticides that can be analyzed using GC-MS/MS official methods and attempted to quantify them using an official LC-MS/MS method. We could not set selected reaction monitoring conditions for 15 of the 31 pesticides due to low ion intensity but conducted recovery tests for the remaining 16 pesticides in brown rice, soybean, peanuts, spinach, cabbage, potato, eggplant, orange, apple, and tea. The 16 pesticides were spiked into homogenized samples at the Japanese maximum residue levels established for each sample type. The coefficient of determination (R^2) values for all the standard calibration curves showed good linearity (R^2>0.9993). The results showed good recoveries for most of the ten tested agricultural products. This study suggests that the 16 selected pesticides suitable for GC-MS/MS analysis can also be quantified using the official LC-MS/MS method.

Keywords: helium shortage, simultaneous analysis, agricultural products

Nabeshi H, Tsutsumi T, Imamura M, Uekusa Y, Hachisuka A, Matsuda R, Teshima R, Akiyama H: Continuous Estimation of Annual Committed Effective Dose of Radioactive Cesium by Market Basket Study in Japan from 2013 to 2019 after Fukushima Daiichi Nuclear Power Plant Accident.

Food Safety 2020;8(4):97-114. doi: https://doi.org/10.14252/foodsafetyfscj.D-20-00017

Radionuclide contamination in foods has been a great concern after the Fukushima Daiichi Nuclear Power Plant (FDNPP) accident. To estimate time trends of daily intake and annual committed effective dose of radionuclides after the accident, radioactive cesium (r-Cs; 134Cs and 137Cs) and potassium-40 (40K) in market basket (MB) samples prepared at 6-month intervals in periods from September 2013 to March 2019 in 15 regions of Japan were analyzed using γ-ray spectrometry. The annual committed effective dose of r-Cs, calculated at non-detected radionuclide levels assumed to be half the limit of detection (LOD), appeared to decrease gradually in 11 regions close to the FDNPP that were more likely to be affected by the accident. Differences in doses among the 15 regions were large just after the accident, but gradually decreased. In particular, 134Cs has not been detected in any MB sample in any region since September 2018, and annual committed effective dose from 134Cs in all regions was mostly constant at around 0.3 μSv/year (given the respective LODs). The maximum annual committed effective dose of r-Cs in this study was decreased from 2.7 μSv/year in September 2013 to 1.0 μSv/year in March 2019. In contrast, the range of annual committed effective dose of 40K varied from approximately 150 to 200 μSv/year during that time frame and did not change much throughout the period of this study. Although annual committed effective doses of r-Cs in regions close to the FDNPP appeared to be higher than in regions far from the FDNPP, doses in all regions are remaining at a much lower levels than the intervention exemption level, 1 mSv/year, in foods in Japan.

Keywords: daily intake, Fukushima Daiichi Nuclear Power Plant accident, radioactive cesium

Matsuo Y[*1], Nakai K[*2], Tatsuta N[*2], Inanami O[*3], Yamamoto K[*3], Mizukawa H[*4], Nagasaka H[*5], Mizutani F[*5], Chisaki Y[*5], Aiba T[*6], Ohba T[*7], Watanabe I[*8], Nabeshi H, Higuchi T[*1], Koga Y[*1], Matsumoto H[*1], Nishimuta K[*1], Miyamoto H[*1], Haraguchi T[*1], Ryuda N[*1], Ueno D[*1]: Using the larvae of caddisfly as a biomonitor to assess the spatial distribution and effective half-life of radiocesium in riverine environments in Fukushima, Japan.

Physics Open 2021;6:100060. doi: https://doi.org/10.1016/j.physo.2021.100060

The environmental monitoring survey using this organisms was called “Caddisfly Watch” and this activity has involved both scientists and local people for collecting them. A simple method is needed for the continuous monitoring of radiocesium (137Cs) contamination in riverine environments after the 2011 accident at the Fukushima Dai-ichi Nuclear Power Plant (FDNPP) in Japan. In a program called “Caddisfly Watch”, we used larvae of the caddisfly Stenopsyche marmorata (Trichoptera: Stenopsychidae) to monitor the spatial distribution and estimate effective half-life (Teff) of 137Cs pollution in riverine environments. Caddisfly larvae showed

that the highest concentration of 137Cs among several aquatic organisms and no apparent variation between growth stage. In addition, caddisfly larvae reflected 137Cs concentrations in suspended particulate matter in their gut, and that showed no seasonal variation, better reproducibility, and significant correlation with those in sediment. Results indicate that caddisfly larvae can be used as a biological sampler of suspended particulate matters. The Teff values of 137Cs concentrations in caddisfly larvae estimated by single component decay function model showed significant fit. The Teff values in Kuma, Maeda, downstream Niida, upstream Niida, Ohkawa, and Ukedo river showed 2.8, 5.7, 3.1, 6.7, 0.6, and 4.8 years (34, 68, 38, 80, 6.9, and 58 months), respectively. The results of declining trend in this study were similar to those in previous reported in Fukushima. Further continuous observations using this simple approach of "Caddisfly Watch" make it possible to predict the future of the contamination with radioactive Cs in the river environment.

Keywords: Radioactive cesium, Fukushima Dai-ichi nuclear power plant, Caddisfly

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Tsutsumi T, Adachi R, Matsuda R, Watanabe T, Teshima R, Akiyama H: Concentrations of polycyclic aromatic hydrocarbons in smoked foods in Japan.
J. Food Prot. 2020;83(4):692-701. doi: https://doi.org/10.4315/JFP-19-486

We evaluated the performance of a GC-MS/MS method for quantifying 16 polycyclic aromatic hydrocarbons (PAHs), which the Scientific Committee on Food and the Joint FAO/WHO Expert Committee on Food Additives have considered to be of concern to human health, and used the method to determine their concentrations in smoked foods. Eighty-seven samples of smoked fish, smoked meat, smoked eggs, dried bonito flakes, and dried bonito-related soup-stock products (disposable powder packets for infusion, instant bouillons, and liquids) were purchased in Japan to analyze their content of the 16 PAHs. Because of the low certainty of some results, the analytical values for some PAHs (e.g. benzo[c]fluorene, chrysene, and dibenzo[a,h]pyrene) are given for informational purposes only. The highest median concentrations of benzo[a]pyrene and the sum of all the 16 PAHs (29 and 760 μg/kg, respectively) were found in the disposable powder packets followed by dried bonito flakes (24 and 512 μg/kg, respectively), and instant bouillons (11 and 227 μg/kg, respectively). These concentrations were much higher than those in the other products tested. We also investigated the percentages of the PAHs transferred from dried bonito flakes and a disposable powder packet to soup stocks commonly prepared at home. These were extremely low (< 4%), even though they contained relatively high concentrations of the PAHs. Finally, the intake of BAP and the sum of the intakes of 4 PAHs, as a marker proposed by the European Food Safety Authority, were estimated based on the data from Japanese food consumption survey and the mean concentrations found in smoked fish and smoked fish products. These estimates suggest intakes of PAHs pose a low concern for consumer health.

Keywords: dried bonito, polycyclic aromatic hydrocarbon, smoked food

Tsutsumi T, Kawashima A*, Hamada N*, Adachi R, Akiyama H: A novel analytical method for determining total polychlorinated biphenyl concentrations in fish and shellfish using a simple and rapid clean-up followed by GC–MS/MS.
J. Food Compos. Anal. 2021;96:103725. doi: https://doi.org/10.1016/j.jfca.2020.103725

We developed an analytical method for determining total (sum of all 209 congeners) polychlorinated

biphenyl (PCB) concentrations in fish and shellfish using a newly developed clean-up method and gas chromatography tandem mass spectrometry. The clean-up involved passing a sample extract through a column containing silica gel and sulfuric-acid-treated silica gel and then a column containing alumina and silver-modified alumina. This gave a short clean-up time and required less organic solvent. No marked losses of any of the 209 PCB congeners were found during clean-up. The trueness and repeatability of the analytical method for total PCBs and seven indicator PCBs in fish and shellfish spiked with a commercial PCB mixture were 92%–94% and 93%–105%, with relative standard deviations of <0.9% and <3.1%, respectively. A certified reference fish sample was analyzed, and the measured concentrations of the certified congeners were within the uncertainty limits of the certified concentrations. The concentration ratios for total PCB and the indicator PCBs in fish determined by the new method and the conventional method using high-resolution gas chromatography high-resolution mass spectrometry were 0.9–1.1 and 0.9–1.2, respectively. The results indicate that the developed method will be very useful for rapidly determining total PCB concentrations in fish and shellfish.

Keywords: polychlorinated biphenyl, fish, clean-up

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赤星千絵*，佐野達哉*，吉田裕一*，橋口成喜*，田口貴章，穐山浩，岡部信彦*：感染性物質を含有する可能性のある人体試料等の理化学試験に関するガイドラインと川崎市健康安全研究所における検討について．

日本食品化学学会誌 2021;28(1):47-53. doi: https://doi.org/10.18891/jjfcs.28.1_47

Prefectural and municipal public health institutes have tested human samples, such as blood and urine, of patients with chemical food poisoning to analyze the harmful substances present in them. Because these samples may contain pathogens, guidelines for their treatment are required to prevent sample-mediated infections in microbiological laboratories. We developed a biosafety guideline for physical and chemical laboratories to establish biosafety strategies, wherein we suggested that the inspection status and sampleborne infection risk in the determination of infectious samples at each institute should be considered. Additionally, we listed the important elements to be considered while establishing the handling and management methods for preventing the accidental exposure to infectious samples while performing physical and chemical experiments. These elements assess the exposure risk of each process in the physical and chemical experiments, select the handling method according to the risk, provide the personnel with instructions on biosafety, consider the infection prevention measures, such as vaccination to the person in charge, supervise and record the performance of inspections, and establish the protocols for dealing with the accident. In this study, the handling and management methods at one institute could be established in accordance with this guideline. In the institute, wet human samples are decided to be treated as infectious samples based on standard precautions, while dry human samples can be treated in the same way as food and environmental samples. The guideline would be useful in treating infectious samples in physical and chemical laboratories.

Keywords：バイオセーフティ，人体試料，感染性試料

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Suzuki Y, Tanaka N*, Akiyama H: Attempt of Bayesian Estimation from Left-censored Data Using the Markov Chain Monte Carlo Method: Exploring Cr(VI) Concentrations in Mineral Water Products.

Food Safety. 2020;8(4):67-89. doi: 10.14252/foodsafetyfscj.D-20-00007

Hexavalent chromium (Cr(VI)) is toxic, carcinogenic, and mutagenic substances. Oral exposure to Cr(VI) is thought to be primarily from drinking water. However, under the certain reporting limit (~0.1 μg/L), percentage of Cr(VI) concentration in mineral water products under the reporting limit were estimated higher than 50%. Data whose values are below certain limits and thus cannot be accurately determined are known as left-censored. The high censored percentage leads to estimation of Cr(VI) exposure uncertain. It is well known that conventional substitution method often used in food analytical science cause severe bias. To estimate appropriate summary statistics on Cr(VI) concentration in mineral

water products, parameter estimation using the Markov chain Monte Carlo (MCMC) method under assumption of a lognormal distribution was performed. Stan, a probabilistic programming language, was used for MCMC. We evaluated the accuracy, coverage probability, and reliability of estimates with MCMC by comparison with other estimation methods (discard nondetects, substituting half of reporting limit, Kaplan-Meier, regression on order statistics, and maximum likelihood estimation) using 1000 randomly generated data subsets (n = 150) with the obtained parameters. The evaluation shows that MCMC is the best estimation method in this context with greater accuracy, coverage probability, and reliability over a censored percentage of 10-90%. The mean concentration, which was estimated with MCMC, was $0.289 \times 10-3$ mg/L and this value was sufficiently lower than the regulated value of 0.05 mg/L stipulated by the Food Sanitation Act.

Keywords: nondetects, left-censored data, Bayesian model

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Okamoto Y, Nunome M, Kondo M, Kitayama I, Suzuki Y, Akiyama H: Quantification of progesterone in beef with marbling using liquid chromatography-tandem mass spectrometry with stable isotope-labelled standards.

Food Addit Contam Part A 2021;38(3):409-417. doi: 10.1080/19440049.2020.1869326

Progesterone (P4) is contained naturally in animal tissue, and it is also used as a veterinary drug in cattle for treatment purposes. To assess the risk from P4 residues in beef derived from treated cattle, it is essential to quantify the P4 contained naturally in cattle tissue (endogenous P4). Therefore, we performed a method validation for the quantification of endogenous P4 (method quantification limit = 0.06 ng g−1) by using isotope-labelled P4s, and investigated the P4 contents in Japanese beef (n= 112; 0.07 to 121 ng g−1). The P4 contents in cattle muscle ranged from 0.07 to 54.3 ng g−1 in males, and from 0.27 to 121 ng g−1 in females. Our investigation also indicated that the developed method using both 13C- and deuterium-labelled P4 standards could be used to certify the recovery of P4 from cattle muscle containing various amounts of intramuscular fat, and enabled the determination of the P4 content in all Japanese beef samples that exceeded the method quantification limit.

Keywords; Progesterone, beef, marbling

Suzuki Y, Matsunaga K*, Yamashita Y*: Assignment of PM2.5 sources in western Japan by non-negative matrix factorization of concentration-weighted trajectories of GED-ICP-MS/MS element concentrations.

Environ. Pollut. 2021;270:116054. doi: 10.1016/j.envpol.2020.116054

Rapid economic growth in Asian countries has raised concerns about the influence of air pollutants transported to Japan by westerly winds. We coupled a gas exchange device (GED) with a tandem inductively coupled plasma mass spectrometer (ICP-MS/MS) to enable direct introduction of PM2.5 to ICP and thus provide better data than could be obtained from samples collected by conventional filter methods. We used the GED-ICP-MS/MS system in Matsue City in western Japan to monitor in real time 29 elements in PM2.5 at 10-min intervals and to estimate the pollutant sources by non-negative matrix factorization (NMF) of concentration-weighted air-mass trajectories. The trajectory analysis identified high V, As, Sn, and Sb concentrations over the ocean from Taiwan to Tsushima Strait. NMF analysis revealed that these elements could be decomposed to multiple factors that indicated a large contribution from oceanic areas. The elemental contributions of these factors were high for metals/metalloids with low melting points as oxides, strongly suggesting that they were sourced from combustion of ship fuel. Our results demonstrate that both emissions from ships at sea and land-based emissions from Japan and continental Asia contribute to PM2.5 in Matsue City.

Keywords: PM2.5, Concentration-weighted trajectory, Non-negative matrix factorization

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Yamakawa H[*1], Nagai H[*2], Ito T[*2], Akiyama H: Development of a simple and reliable high-performance liquid chromatography–tandem mass spectrometry approach to simultaneously detect grains specified in food allergen labeling regulation on processed food commodities.
J Chromatogr A. 2020; 1639: 461877. doi: 10.1016/j.chroma.2021.461877

An analytical approach using high-performance liquid chromatography–tandem mass spectrometry (LC–MS/MS) was developed to simultaneously detect Fagopyrum esculentum Moench (buckwheat) and cereals containing gluten (Triticum species including wheat and spelt, rye, barley, and oats) that were specified in regulations for food allergen labeling on processed foods. Trypsin-digested peptides were purified from different processed food commodities and heptapeptides derived from buckwheat 13S globulin (GFIVQAR, *m/z* 395.8 [precursor] > 177.0 [product]) and Triticum low molecular weight glutenin (QIPEQSR, *m/z* 429.3 [precursor] > 616.2 [product]) were specifically detected each species at levels as low as 0.050–0.056 μg/L and 0.028–0.032 μg/L, respectively. Detection of these synthetic peptides was quantitative to over 100 μg/L by reference to the synthetic peptide calibration curves and at recovery rates, 76.6 ± 4.1%–104.8 ± 17.1% and 82.4 ± 2.0%–105.8 ± 5.3%, for GFIVQAR and QIPEQSR, respectively, when 1–1,000 μg of these peptides were spiked into a retort tomato sauce for pasta or dried instant soup. In combination with LC–MS/MS detection methods specific to other cereals containing gluten (rye, barley, and oats), the developed analytical approach was applicable to a wide variety of processed food commodities for food allergen labeling.

Keywords: LC–MS/MS, Gluten, Fagopyrum esculentum Moench (buckwheat)

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井上智子[*1]，堀口逸子[*2]，平原嘉親[*3]，穐山浩：登録検査機関の業務管理の向上に関する調査研究.
日本食品化学学会誌 2020;27:190-195. doi: https://doi.org/10.18891/jjfcs.27.3_190

登録検査機関の業務管理向上を目的として，2013年から2019年の6年間の厚生局の指導事項を調査した．指摘事項の中で試薬，精度管理，機械器具の管理に関する指導の頻度が高く，登録検査機関で業務管理を改善する上で重要な事項であり，登録検査機関が注意すべき課題であることを明らかにした．また，厚生局の立入検査時の指導の改善点，厚生労働省からの通知，ガイドラインの改善等を明らかにした．本研究では，登録検査機関の業務管理の問題点に対する具体的な改善案を提案した．その提案により登録検査機関の業務管理の向上に期待される．

Keywords：登録検査機関，業務管理，GLP

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Igarashi Y*, Takahashi M*, Tsutsumi T, Inoue K*, Akiyama H: Monitoring Analysis of Perfluoroalkyl Substances and F-53B in Bottled Water, Tea and Juice Samples by LC-MS/MS.
Chem Pharm Bull 2021;69:286-290. doi: https://doi.org/10.1248/cpb.c20-00888

Monitoring analysis of 14 per- and polyfluoroalkyl substances (PFAS), 9-chlorohexadecafluoro-3-oxanonane-1-sulfonate (F-53B) and dodecafluoro-3H-4,8-dioxanonanoate (ADONA) in bottled drinking water, tea and juice samples was performed using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) and solid-phase extraction (SPE). In the electrospray negative ion mode, the limit of detection and limit of quantification (LOQ) values were 0.1 to 0.8 ng/mL and 0.2 to 1.6 ng/mL, respectively. The calibration curves were linear from LOQ to 50 ng/mL (r^2 > 0.999). The SPE procedure (Presep PFC-II) was utilized for sample preparation and recovery rates for three standards (35, 70 and 140 ng/L) were 80.4–118.8% with RSD ≤ 0.6%. Using the developed method, various samples (n=54) from Japanese markets were investigated for PFAS and F-53B contamination, and values below the LOQ were observed. It is concluded that for monitoring products in the Japanese market, our method represents a significant improvement over complex techniques for the quantification of PFAS and related compounds from various foods.

Keywords: per- and polyfluoroalkyl substances (PFAS), liquid chromatography tandem mass spectrometry, drinking water

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Takahashi M*, Yada M*, Morimoto K*, Nemoto S, Akiyama H, Inoue K*: Simultaneous determination of alachlor and its metabolites in bovine tissues, milk and egg by liquid chromatography-tandem mass spectrometry.

SSC plus 2020;4:68-76. doi: https://doi.org/10.1002/sscp.202000091

Alachlor is a chloroacetanilide herbicide used to control annual grasses in grazing system and has been observed along with its metabolites in animal foodstuffs. The regulatory gas chromatographic techniques for multiresidue monitoring methodology of herbicides in agricultural crops are deficient in recovery and nonspecific for its hydrophilic metabolites. Alachlor residue violations in animal foodstuffs are increasing significantly and therefore, an improved method is required. In this study, a rapid, useful, routine and sensitive liquid chromatography with tandem mass spectrometry for simultaneously determining and confirming alachlor and its metabolites (2-chloro-N-(2,6-diethylphenyl) acetamide, 2,6-diethylaniline, and 2-ethyl-6-(1-hydroxyethyl) aniline) in bovine tissues, milk and egg samples was examined. The proposed method uses a simple extraction with methanol followed by QuEChERS and solid-phase extraction cleanup. Observably, calibration curves were linear from LOQ to 100 ng/mL ($r^2 > 0.999$). The recovery values from four different fortified at 0.02 and 0.2 mg/g ranged from70.4%–107.6% with RSD ≤ 7.1%. The developed analytical process can be used to monitor alachlor residue in animal foodstuffs.

Keywords: Alachlor, bovine tissues, liquid chromatography with tandem mass spectrometry

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日本食品化学学会誌 2020;27:141-8. doi: https://doi.org/10.18891/jjfcs.27.3_141

The Japan Flavor & Fragrance Materials Association (JFFMA) has conducted poundage surveys of flavoring substances used in Japan in 2001, 2005, 2010 and 2015. The number of flavoring substances used in Japan was the maximum in 2001 and has been decreasing thereafter. The reason for this decline is thought to be the discontinuation of use of flavoring substances that were used only in Japan in response to the globalization of flavor regulations and changes in consumer preferences, resulting in increased unification with internationally accepted flavoring items. The estimated daily intake of flavoring substances was calculated by the MSDI method, and the number of flavoring substances for each range of estimated intake was compared. It is generally said that "the characteristics of flavor ingredients are small amounts and multi-components" and this was verified by the four poundage surveys in Japan. In order to provide up-to-date exposure data for scientific safety assessment, it is desirable that poundage surveys of flavoring substances are conducted regularly.

Keywords：使用量調査，食品香料化合物，推定摂取量

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食品衛生学雑誌 2020;61:53-7. doi: https://doi.org/10.3358/shokueishi.61.53

加工食品にはビタミンDとして食品添加物のエルゴカルシフェロール（D_2）およびコレカルシフェロール（D_3）が使用されており，加工食品中の含有量把握のため検証された分析法が必要とされる．本研究ではD_2とD_3を分離定量する方法として，栄養成分分析に用いられる日本食品標準成分表2015年版〈七訂〉分析マニュアルのビタミンD分析法（マニュアル法）の加工食品中のビタミンD分析への適用性について検討を行った．検討の結果，マニュアル法にいくつかの課題が認められたため，マニュアル法に改良を加えて加工食品への適用性を検討した．野菜ジュース，豆乳，コーンフレークを用いた添加回収実験の結果，改良マニュアル法は推定方法定量下限（EMLOQ）相当量での回収率（相対標準偏差）がD_2で103～112%（4.7～12.6%），D_3で102～109%（2.4～21.8%），EMLOQの10倍量添加ではD_2で100～110%

（4.0～7.4%），D_3では102～105%（3.8～4.8%）であった．この結果から，改良マニュアル法は加工食品中のD_2，D_3分析に適用可能な真度および精度を有すると推察された．

Keywords：食品添加物分析，エルゴカルシフェロール，コレカルシフェロール

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Nishizaki Y, Ishizuki K, Masumoto N, Tada A, Sugimoto N, Sato K: HPLC determination of quercetin using relative molar sensitivity to methylparaben as a single reference.
日本食品化学学会誌 2020;27:42-52. doi: https://doi.org/10.18891/jjfcs.27.2_42

A simple and reliable high-performance liquid chromatography (HPLC) method for the determination of quercetin (QR) was developed. The method requires no QR reference; instead, it uses relative molar sensitivity (RMS) to a single reference, methylparaben (MPB). The RMS of QR/MPB was determined using an offline combination of ^{1}H-quantitative NMR (^{1}H-qNMR) and HPLC/UV or HPLC/photodiode array (PDA). In this study, the RMS represents the sensitivity ratio of QR to MPB per mole under defined HPLC conditions. The QR in two natural food additives was quantified using three different techniques such as ^{1}H-qNMR, UV–Vis spectrophotometry, and our single-reference HPLC method. Based on certain amounts of QR in two food additive products, the similar results using ^{1}H-qNMR (mass fraction: 96.9% and 96.1%) and single-reference HPLC (95.8% and 95.0%) were exhibited. On the other hand, the results using UV–Vis spectrophotometry (98.5% and 99.2%) without separation of QR and others (kaempferol and isorhamnetin impurities) showed slightly higher amounts than other techniques. In these techniques, the single-reference HPLC is advanced for the simple and accurate quantification of QR in food additive products without native standard.
Keywords: quercetin, relative molar sensitivity, quantitative NMR

西﨑雄三，石附京子，増本直子，杉本直樹，佐藤恭子：既存添加物である精油除去ウイキョウ抽出物中に含まれる主成分の抗酸化能評価．
日本食品化学学会誌 2020;27:164-72. doi: https://doi.org/10.18891/jjfcs.27.3_164

Essential oil-removed fennel extract, which is used as an antioxidant agent derived from natural source, was analyzed by using HPLC-PDA/MS. The main constituents of the commercial products were sinapyl alcohol 4-O-β-D-glucoside (syringin), quercetin 3-O-β-D-glucuronide (Q3GA), and acyl-quinic acids, such as 5-O-caffeoylquinic acid (chlorogenic acid), 3-O-caffeoylquinic acid, 4-O-caffeoylquinic acid, and 3,5-di-O-caffeoylquinic acid. The reagents of these main constituents were subjected to external calibration quantitative ^{1}H-NMR. There were approximately 10% differences in the absolute purities between the reagents. By reflecting the absolute purities to each calibration curve, a reliable HPLC quantification showed that the contents of main constituents in the products were 0.05%–0.2%. Furthermore, the antioxidant activities of the products and the main constituents were evaluated by antioxidant assay: 1,1-diphenyl-2-picrylhydrazyl (DPPH) and thiocyanate methods. Among the main constituents, Q3GA and chlorogenic acids were responsible to the antioxidative potency of the products.
Keywords：ウイキョウ，DPPH法，外部標準法定量NMR

Nishizaki Y, Lankin DC*, Chen SN*, Pauli GF*: Accurate and precise external calibration enhances the versatility of quantitative NMR (qNMR).
Anal. Chem., 2021;93:2733-41. doi: 10.1021/acs.analchem.0c02967

Quantitative ^{1}H nuclear magnetic resonance (qHNMR) is a highly regarded analytical methodology for purity determination as it balances metrological rigor, practicality, and versatility well. While ideal for intrinsically mass-limited samples, external calibration (EC) qHNMR is overshadowed by the prevalence of internal calibration and perceived rather than real practical limitations. To overcome this hurdle, this study applied the principle of reciprocity, certified reference materials (caffeine as analyte, dimethyl sulfone as calibrant), and a systematic evaluation of data acquisition workflows to extract key factors for the achievement of accuracy and precision in EC-qHNMR. Automatic calibration of the 90° pulse width (90 PW) formed the foundation for the

principle of reciprocity and used optimized nutation experiments, showing good agreement with values derived from manual high-precision measurement of 360 PW. Employing the automatic 90 PW calibration, EC-qHNMR with automatic vs manual tuning and matching (T&M) yielded the certified purity value within 1% error. The timing of T&M (before vs after shimming) turned out to be critically important: sufficient time is required to achieve full-temperature equilibrium relative to thermal gradients in the air inside the probe and the sample. Achievable accuracy across different NMR solvents varies with differences in thermal conductivity and leads to 2% or greater errors. With matching solvents, the demonstrated accuracy of ～1.0% underscores the feasibility of EC-qHNMR as a highly practical research tool.

Keywords: qNMR, external calibration, the principle of reciprocity

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Masumoto N, Ishizuki K, Nishizaki Y, Ohtsuki T[*1], Kuroe M[*2], Yamazaki T[*2], Numata M[*2], Matsufuji H[*1], Sugimoto N, Sato K: Determination of mogroside V in luohanguo extract for daily quality control operation using relative molar sensitivity to single-reference caffeine.

Chem. Pharm. Bull., 2021;69:18-25. doi: https://doi.org/10.1248/cpb.c20-00245

Mogroside V is one of the characteristic and effective components of luohanguo extract, a food additive used as a sweetener in Japan as per Japan's Standards and Specifications for Food Additives (JSFA; 9th ed.). JSFA stipulates that the quantitative determination for mogroside V content in luohanguo extract applies HPLC using analytical standard mogroside V. However, no mogroside V reagents with proven purities are commercially available. Therefore the current JSFA determination method is not particularly suited for daily quality control operations involving luohanguo extract. In this study, we applied an alternative quantitative method using a single reference with relative molar sensitivity (RMS). It was possible to calculate the accurate RMS by an offline combination of ^{1}H-quantitative NMR spectroscopy (^{1}H-qNMR) and an HPLC/variable-wavelength detector (VWD). Using the RMS of mogroside V to a commercial certified reference material grade caffeine, the mogroside V contents in luohanguo extracts could be determined using HPLC/VWD without analytical standard mogroside V. There was no significant difference between the mogroside V contents in luohanguo extracts determined using the method employing single-reference caffeine with the RMS and using the JSFA method. The absolute calibration curve for the latter was prepared using an analytical standard mogroside V whose purity was determined by ^{1}H-qNMR. These results demonstrate that our proposed method using a single reference with RMS is suitable for quantitative determination of mogroside V in luohanguo extract and can be used as an alternative method to the current assay method in JSFA.

Keywords: relative molar sensitivity (RMS), single-reference HPLC, mogroside V

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Horiuchi R*, Nishizaki Y, Okawa N*, Ogino A*, Sasaki N*: Identification of the biosynthetic pathway for anthocyanin triglucoside, the precursor of polyacylated anthocyanin, in red cabbage.

J. Agric. Food Chem., 2020;68:9750–8. doi: 10.1021/acs.jafc.0c03480

Red cabbage anthocyanin is utilized as a natural food colorant because of its stable and brilliant coloration. The major anthocyanin of red cabbage is cyanidin (Cy) mono- and di-acyltriglucoside; however, the biosynthetic pathway to generate this anthocyanin remains unclear. We isolated and identified four uridine diphosphate-glucose-dependent glucosyltransferase (UGT) cDNAs from red cabbage using RNA-seq. UGTs are involved in Cy triglucoside (CytriG) synthesis, the precursor of Cy acyltriglucoside. Enzymatic assays using recombinant proteins suggested that UGT78D5 encodes Cy 3GT, UGT79B45 encodes Cy 3-glucoside GT, UGT75C2 encodes Cy 3-sophoroside (Cy3Sp) 5GT, and UGT79B44 encodes flavonol 3-glucoside GT. Anthocyanin GT assays using crude proteins prepared from red cabbage suggested that CytriG is produced from intermediate products in the following order: Cy, Cy3G, Cy3Sp, and CytriG.

Keywords: red cabbage, glucosyltransferase,

anthocyanin

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Miura T*[1], Sugimoto N, Bhavaraju S*[2], Yamazaki T*[3], Nishizaki Y, Liu Y*[2], Bzhelyansky A*[2], Amezcua C*[4,5], Ray J*[4,6], Zailer E*[7], Diehl B*[7], Gallo V*[8], Todisco S*[8], Ofuji K*[9], Fujita K*[10], Higano T*[11], Geletneky C*[12], Hausler T*[12], Singh N*[12], Yamamoto K*[13], Kato T*[13], Sawa R*[14], Watanabe R*[15], Iwamoto Y*[1], Goda Y: Collaborative study to validate purity determination by ^{1}H quantitative NMR spectroscopy by using internal calibration methodology.

Chem. Pharm. Bull., 2020;68:868-878. doi: 10.1248/cpb.c20-00336

NMR spectroscopy has recently been utilized to determine the absolute amounts of organic molecules with metrological traceability since signal intensity is directly proportional to the number of each nucleus in a molecule. The NMR methodology that uses hydrogen nucleus (^{1}H) to quantify chemicals is called quantitative ^{1}H-NMR (^{1}H qNMR). The quantitative method using ^{1}H qNMR for determining the purity or content of chemicals has been adopted into some compendial guidelines and official standards. However, there are still few reports in the literature regarding validation of ^{1}H qNMR methodology. Here, we coordinated an international collaborative study to validate a ^{1}H qNMR based on the use of an internal calibration methodology. Thirteen laboratories participated in this study, and the purities of three samples were individually measured using ^{1}H qNMR method. The three samples were all certified via conventional primary methods of measurement, such as butyl p-hydroxybenzoate Japanese Pharmacopeia (JP) reference standard certified by mass balance; benzoic acid certified reference material (CRM) certified by coulometric titration; fludioxonil CRM certified by a combination of freezing point depression method and ^{1}H qNMR. For each sample, ^{1}H qNMR experiments were optimized before quantitative analysis. The results showed that the measured values of each sample were equivalent to the corresponding reference labeled value. Furthermore, assessment of these ^{1}H qNMR data using the normalized error, E_n-value, concluded that statistically ^{1}H qNMR has the competence to obtain the same quantification performance and accuracy as the conventional primary methods of measurement.

Keywords: collaborative study, measurement uncertainty, quantitative (q)NMR

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Yoshida K*, Teppabut Y*, Sawaguchi R*, Nakane Y*, Hayashi E*, Oyama K*, Nishizaki Y, Goda Y, Kondo T*: 5,7,3',4'-Tetrahydroxyflav-2-en-3-ol 3-*O*-glucoside, a new biosynthetic precursor of cyanidin 3-*O*-glucoside in the seed coat of black soybean, *Glycine max*.

Sci. Rep., 2020;10(171834):1-10. doi: https://doi.org/10.1038/s41598-020-74098-6

The seed coat of mature black soybean, *Glycine max*, accumulates a high amount of cyanidin 3-*O*-glucoside (Cy3G), which is the most abundant anthocyanin in nature. In the pod, it takes two months for the seed coat color change from green to black. However, immature green beans rapidly adopt a black color within one day when the shell is removed. We analyzed the components involved in the color change of the seed coat and detected a new precursor of Cy3G, namely 5,7,3',4'-tetrahydroxyflav-2-en-3-ol 3-*O*-glucoside (2F3G). Through quantitative analysis using purified and synthetic standard compounds, it was clarified that during this rapid color change, an increase in the Cy3G content was observed along with the corresponding decrease in the 2F3G content. Chemical conversion from 2F3G to Cy3G at pH 5 with air and ferrous ion was observed. Our findings allowed

us to propose a new biosynthetic pathway of Cy3G via a colorless glucosylated compound, 2F3G, which was oxidized to give Cy3G.

Keywords: biosynthetic pathway, anthocyanin, black soybean

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Ohtsuki T*, Matsuoka K*, Fuji Y*, Nishizaki Y, Masumoto N, Sugimoto N, Sato K, Matsufuji H*: Development of an HPLC method with relative molar sensitivity based on ^{1}H-qNMR to determine acteoside and pedaliin in dried sesame leaf powders and processed foods.

PLoS ONE, 2020;e0243175:1-12. doi: https://doi.org/10.1371/journal.pone.0243175

A high-performance liquid chromatography (HPLC) method with relative molar sensitivity (RMS) based on ^{1}H quantitative NMR spectroscopy (^{1}H-qNMR) has been developed for food ingredients such as acteoside (verbascoside) and pedaliin (pedalitin-6-O-glucoside) without requiring authentic and identical standards as the reliable analytical methods. This method is used methyl 4-hydroxybenzoate (MHB) as an alternative reference standard. Each RMS is also calculated from the ratio of each analyte's molar absorption coefficient to that of MHB after correcting the purities of the analytes and reference standard by ^{1}H-qNMR. Therefore, this method can quantify several analytes with metrological traceability to the International System of Units (SI) using the RMS and one alternative reference standard. In this study, the content of acteoside and pedaliin in several samples, such as dried sesame leaf powders and commercially processed foods, can be determined by the proposed RMS method and demonstrated in good agreement that obtained by a conventional method. Moreover, the proposed method yields analytical data with SI-traceability without the need for an authentic and identical analyte standard. Thus, the proposed RMS method is a useful and practical tool for determining acteoside and pedaliin in terms of the accuracy of quantitative values, the routine analysis, and the cost of reagents.

Keywords: acteoside, ^{1}H-qNMR, relative molar sensitivity

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酒井有希*，増本直子，西﨑雄三，大槻崇*，松藤寛*，杉本直樹，佐藤恭子：相対モル感度を用いたsingle-reference HPLC法が定量値に影響を及ぼす要因の検討と機能性表示食品中のルテイン定量への応用.

日本食品化学学会誌 2020;27:123-34. doi: https://doi.org/10.18891/jjfcs.27.3_123

Chromatographic techniques, such as high-performance liquid chromatography (HPLC), are typically adopted as quantitative methods for the separation and identification of functional substances classed as Food with Function Claims (FFC). Although the quantitative values determined by chromatographic approaches are ensured by using high purity reference materials, for most functional substances, such references are not commercially available. In this study, we applied an alternative quantitative technique using the relative molar sensitivity (RMS), namely single-reference HPLC, to quantitatively analyze the content of lutein in FFC. In this method, RMS of the analyte to a single reference compound was used to determine the analyte content in FFC using HPLC/ photodiode array detector (PDA). Notably, the approach did not require the use of any reference materials. Since there were no researches on factors affecting on quantitative value by single-reference HPLC method, the concentration ranges of the analyte and single reference, which yielded reliable RMS values, were comprehensively evaluated to obtain accurate quantitative values by single-reference HPLC. Consequently, when the signal to noise ratio of the peaks corresponding to the single reference and analyte was above 50, the differences in the quantitative values were within approximately 1.5%. Based on the obtained results, RMS of lutein to a single reference compound, specifically sudan I, which is a stable commercially available analytical standard, was established at 8.18. It was found that there was no significant difference between the lutein content of FFC determined utilizing the single-reference HPLC method and the absolute calibration curve approach. The calibration curve was generated using lutein with adjusted purity measured by ^{1}H-qNMR. The outcomes of the study demonstrate that lutein in FFC can be indirectly quantified employing inexpensive and high-purity sudan I as a single reference.

Keywords：ルテイン，機能性表示食品，相対モル感度

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Kuroe M*, Numata M*, Masumoto N, Nishizaki Y, Sugimoto N, Itoh N*: Use of relative molar sensitivity as a specific value for evaluating heptaoxyethylene dodecyl ether concentrations in methanol solution.

Anal. Sci., [Epub ahead of print] doi: 10.2116/analsci.20N031

Relative molar sensitivity (*RMS*) determined using quantitative ^{1}H NMR and HPLC with a refractive index (RI) detector was applied as a specific value for quantifying the levels of heptaoxyethylene dodecyl ether (HOEDE), a typical non-ionic surfactant, in methanol solutions. *RMS* was robust against changes of analytical conditions (i.e., RI cell temperature, acetonitrile content in the mobile phase, HPLC system). Furthermore, the obtained HOEDE concentrations using a previously evaluated RMS were comparable to those obtained using a reference method over 1 year.

Keywords: relative molar sensitivity (RMS), qNMR, heptaoxyethylene dodecyl ether

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Takahashi M*, Nishizaki Y, Masumoto N, Sugimoto N, Sato K, Inoue K*: Quantification of tea-derived catechins without the requirement for respective calibration curves by single reference liquid chromatography based on relative molar sensitivity.

J. Sci. Food Agric., [Epub ahead of print] doi: https://doi.org/10.1002/jsfa.11013

Many studies report the monitoring of catechins in tea samples by chromatographic techniques. Unfortunately, only a small number of screening assays for catechins exist as a result of the complexity of authentic standards for the respective calibration curves. In the present study, a single reference (SR) exhaustive assay for the simultaneous quantification of tea-derived catechins by liquid chromatography (LC) with photodiode array and fluorescence detectors based on relative molar sensitivity (RMS) was developed as a screening assay of common tea samples without respective calibration curves using authentic standards. Three original SR standards were proposed based on flavonoid structures, evaluated by quantitative ^{1}H-NMR based on an indirect standard (1,4-bis(trimethylsilyl) benzene-d_4) and successfully separated in a LC chromatogram. In tea samples with these added SR calculated based on RMS, the concentrations of eight tea-derived catechins could be measured with a relative SD of < 8.5% by a single LC run. This LC screening assay based on RMS allows reliable quantification without the requirement for respective calibration curves using authentic standards.

Keywords: relative molar sensitivity (RMS), single-reference HPLC, catechins

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Abe Y, Kobayashi N, Yamaguchi M, Mutsuga M, Ozaki A*, Kishi E*, Sato K: Determination of formaldehyde and acetaldehyde levels in poly (ethylene terephthalate) (PET) bottled mineral water using a simple and rapid analytical method.

Food Chemistry, 2020;344:128708 doi: https://doi.org/10.1016/j.foodchem.2020.128708

A simple and rapid analytical method was developed for the determination of formaldehyde (FA) and acetaldehyde (AA) contents in water. FA and AA were derivatized by 2,4-dinitrophenylhydrazine in an LC vial for 20 min at room temperature, about 25℃ , and analyzed using LC–MS/MS. The calibration curve exhibited excellent linearity for FA and AA concentrations of 2–150 ng/mL. Recovery tests using ultra-pure water and commercially available PET-bottled mineral water samples showed good trueness and precision. We determined the FA and AA contents in 105 PET-bottled mineral water samples on the Japanese market using this method. FA was detected in 61% of the samples at levels from 2.6 to 31.4 ng/mL, while AA was detected in 68% at levels from 5.3 to 143.5 ng/mL. These results demonstrate that the concentrations of FA and AA in PET-bottled mineral water on the Japanese market have not changed significantly over the last decade.

Keywords: polyethylene terephthalate, formaldehyde, acetaldehyde

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阿部裕，山口未来，大野浩之*1，阿部智之*2，六鹿元雄，佐藤恭子：ポリスチレン製食品用器具・容器包装の揮発性物質試験におけるスチレンのキャリーオーバーの低減化に関する検討.
日本食品化学学会誌 2020; 27: 173-7. doi: https://doi.org/10.18891/jjfcs.27.3_173

ポリスチレン製食品用器具・容器包装の揮発性物質試験において発生するスチレン（ST）のキャリーオーバーの低減化について検討した．GC測定条件の昇温プログラムの変更，洗浄溶液の測定，シリンジ洗浄では低減効果はほとんど得られなかった．シリンジ交換ではわずかに低減した．一方，ライナー内の石英もしくはガラスウールを交換することで完全に除去することができた．したがって，STのキャリーオーバーの主な原因はライナー内のウールに蓄積したSTオリゴマーやポリマーであると考えられた．キャリーオーバーによるST量は規格値の1/10以下であるため，適否判定に影響を及ぼすことはほとんどないと考えられた．しかし，厳密な定量を行うためには，キャリーオーバーがないことを測定前に確認する必要がある.
Keywords：スチレン，キャリーオーバー，揮発性物質試験

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阿部裕，山口未来，大野浩之*1，阿部智之*2，六鹿元雄，佐藤恭子：ナイロン製食品用器具・容器包装のカプロラクタム試験におけるピーク形状改善のためのGC測定条件の検討.
日本食品化学学会誌 2020; 27:178-183. doi: https://doi.org/10.18891/jjfcs.27.3_178

ナイロン製食品用器具・容器包装のカプロラクタム（CPL）試験において発生するCPLのピーク分離などのピーク形状悪化現象の原因を推測するとともに，GC測定条件を変更し，ピーク形状が改善されるかを検討した．CPLのピーク形状悪化現象は，試験溶液をGCに注入する際のオーバーロードが原因であると推測された．そこで，カラムサイズ，注入量，スプリット比，注入口温度，オーブン昇温条件，ライナー種類もしくはグラスウール量の変更および試験溶液の有機溶媒の希釈によるピーク形状の改善効果を検討した．その結果，注入口温度をカラムの最高耐熱温度である280℃に設定し，さらに試験溶液の注入量を半分にするか，もしくは注入口温度を280℃に設定し，さらに試験溶液をエタノールなどの有機溶媒で倍に希釈したものを注入することでもピーク形状の大幅な改善が確認された．また内標準物質として添加したヘプタラクタム（HPL）は，クロマトグラム上でのピーク形状がCPLと同一の挙動を示した．したがって，HPLは内標準物質本来の試験溶液の注入誤差の補正という目的以外に，CPLの分離を確認する指標物質としても有用であることが示唆された.
Keywords：カプロラクタム，ポリアミド，水素炎イオン化検出器付きガスクロマトグラフ

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片岡洋平，竹内温教*，小林尚*，菊川浩史*，佐藤恭子，穐山浩：2018年のミネラルウォーター類製品における六価クロムの含有実態調査.
食品衛生学雑誌 2020; 61:72-76. doi: https://doi.org/10.3358/shokueishi.61.72

2018年にわが国の市場から購入したミネラルウォーター類（MW）155製品中の六価クロム濃度をポストカラム誘導体化法を用いたイオンクロマトグラフィーにより実態調査した．実態調査の分析と併行して行った155製品の添加試料の分析から95-106％の範囲で回収率が得られ，規格値への適合判定を行うための分析が適正に実施されたと評価した.

調査した155製品のうち54製品から六価クロムが定量下限値（0.0001 mg/L）を上回る濃度で検出され，検出率は35％となった．また，検出された濃度の最小値は0.0001 mg/L，最大値は0.045 mg/L，中央値は0.0003 mg/Lであった．0.0001-0.0002 mg/Lの範囲で六価クロムが検出される製品数が最も多かった．現状の食品衛生法により設定されているMW中の規格値（0.05 mg/L）を超過する濃度で検出された製品はなかった.
Keywords：六価クロム，イオンクロマトグラフィー，ミネラルウォーター

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片岡洋平，渡邉敬浩，鴇田敦*，近藤翠，滝澤和宏*，佐藤恭子，穐山浩：ミネラルウォーター類製品におけるフタル酸ジ-2-エチルヘキシルの含有実態調査.
日本食品化学学会誌 2020; 27:118-122. doi: https://doi.org/10.18891/jjfcs.27.2_118

2018年に国内流通していたミネラルウォーター類（MW）155製品中のフタル酸ジ-2-エチルヘキシル（DEHP）濃度をGC-MS法を用いて実態調査した．調査に先立ち，使用したGC-MS法を性能評価し，妥当性を

確認した．また，実態調査した製品から無作為に選択した20製品の一部を採取しDEHPを添加して調製した添加試料を分析した．回収率は93-99％の範囲で得られ，MW製品への分析法の適用性が確認された．妥当性確認された分析法を用いて調査した全155製品から，定量下限値（0.007 mg/L）を上回る濃度でDEHPは検出されなかった．この結果により，調査した155製品中のDEHP濃度が，WHO飲料水水質ガイドライン値（0.008 mg/L）を下回っていることが確認された．

Keywords：フタル酸ジ-2-エチルヘキシル，ミネラルウォーター，実態調査

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Yamamoto S, Kitagawa W*1, Nakano M*1, Asakura H, Iwabuchi E*2, Sone T*1, Asano K*1: Plasmid sequences of four large plasmids carrying antimicrobial resistance genes in *Escherichia coli* strains isolated from beef cattle in Japan.

Microbiology Resource Announcements 2020;9(20):e00219-20. doi:10.1128/MRA.00219-20

Escherichia coli is a common reservoir for antimicrobial resistance genes that can be easily transformed to possess multidrug resistance through plasmid transfer. To understand multidrug resistance plasmids, we report the plasmid sequences of four large plasmids carrying a number of genes related to antimicrobial resistance that were found in E. coli strains isolated from beef cattle.

Keywords: antimicrobial resistance gene, *Escherichia coli*, beef cattle

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Montojo MU*, Tanyag EB*, Perelonia SKB*, Cambia DF*, Oshiro N: Ciguatera in the Philippines: Examining reef fish vectors and its causative benthic dinoflagellates in Visayan and Sibuyan Seas.

Philipp J Fisher. 2020;27(1):19-29. doi:10.31398/tpjf/27.1.2019A0015

Ciguatera Fish Poisoning (CFP) is primarily caused by ingesting reef fishes contaminated with ciguatoxins (CTX) produced by the *Gambierdiscus* species. The unpredictability of this type of food poisoning poses risks to public health and adversely affecting the fish trade industry. This study aimed to provide useful information on ciguatera in the Philippines. Different reef fish species and host-macroalgae for benthic dinoflagellates were collected in Visayan and Sibuyan Seas. Ciguatoxins were extracted from reef fish samples, and toxicity was determined qualitatively using mouse bioassay. Meanwhile, cell density estimation of toxic benthic dinoflagellates isolated from the host-macroalgae was done through microscopy. It was observed that 4.46% of the total reef fish samples were positive with ciguatoxins. Spatially, Carles, Iloilo in Visayan sea had the highest number of toxic specimens belonging to *Epinephelus merra, Lethrinus lentjan, Lutjanus campechanus, Scarus quoyi, Siganus guttatus,* and *Sphyraena barracuda*. Based on data gathered from three sampling sites, fish toxin occurrence is observed to be site-specific. Geographical conditions affect the frequency of toxic samples. Moreover, fish weight is not a good predictor of fish toxicity. For toxic benthic dinoflagellates, *Gambierdiscus* spp. were observed to have the lowest cell density count among other dinoflagellates averaging 7-115 cells per 100 g macroalgae. On the other hand, *Ostreopsis* spp. had the highest average cell density of 118-1,455 cells per 100 g macroalgae, followed by *Prorocentrum* spp. (207-594 cells per 100 g macroalgae). Fish toxicity is directly proportional to the occurrence of benthic dinoflagellates in areas as seen during dry season. Monitoring and management of CFP on identified reef fish vectors and its causative benthic dinoflagellates in the area are necessary to promote food safety and fair trade practice.

Keywords: ciguatoxin, *Gambierdiscus*, toxic reef fish

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Asakura H, Sakata J*1, Yamamoto S, Igimi S*2: Draft genome sequences of non-H_2S-producing strains of *Salmonella enterica* serovars Infantis, Enteritidis, Berta, and Kiambu in Japan.

Microbiology Resource Announcements 2020;9(30):e00335-20. doi:10.1128/MRA.00335-20

We report the draft genome sequences of six strains of *Salmonella enterica* serovars Berta, Enteritidis, Infantis, and Kiambu, isolated from humans or chicken meats in Osaka, Japan, that were negative for hydrogen sulfide production. Their genome sizes

ranged from 4,460,389 to 4,933,483 bp, with 3 to 9 rRNAs and 64 to 73 tRNAs and with coverages of 95 × to 159×.

Keywords: *Salmonella enterica*, H_2S, genome sequence

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Sugiyama H*1, Morishima Y*1, Kagawa C*1, Araki J*1, Iwaki T*2, Ikuno H*3, Miguchi Y*3, Komatsu N*4, Kawakami Y*5, Asakura H: Current Incidence and Contamination Sources of Ascariasis in Japan.

Shokuhin Eiseigaku Zasshi 2020;61(4):103-108. doi: 10.3358/shokueishi.61.103

Ascaris lumbricoides or roundworm is one of the key soil-transmitted helminths affecting humans. A small number of infections continue to occur in Japan, suggesting plant foodstuff contamination as the source of infection. To understand the current status of ascariasis incidence and to identify potential sources of infection, we extensively surveyed the available literature and collected data from testing facilities that examined clinical samples or foodstuffs. We observed that from 2002 onwards, there was a decrease in the number of ascariasis cases reported in scientific journals. Data from a clinical testing facility indicated that the number of detected cases declined remarkably from 2009. Foodstuff testing facilities reported that 11 of 10,223 plant foodstuff specimens were contaminated with anisakid nematodes but not with *Ascaris*. Imported kimchi was suspected as the most probable source of ascarid nematode infection, as one *Ascaris* egg-positive sample was detected among 60 kimchi samples in a testing facility. Therefore, the sources of *Ascaris* infection are still not fully known and need to be clarified to establish preventive countermeasures to safeguard *Ascaris* infections that continue to occur in Japan.

Keywords: *Ascaris* infection, plant foodstuff, incidence

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Sasaki Y, Asai T*1, Haruna M*2, Sekizuka T*3, Kuroda M*3, Yamada Y*2: Isolation of methicillin-resistant *Staphylococcus aureus* ST398 from pigs in Japan.

Japanese Journal of Veterinary Research 2020;68(3):197-202. doi:10.14943/jjvr.68.3.197

Two investigations were conducted to confirm the presence of MRSA ST398 in domestic Japanese pigs. In the first investigation, nasal swabs were collected from 500 pigs on 50 pig farms between August 2012 and February 2013. MRSA ST398 was isolated from four pigs from a farm. In the second investigation, nasal swabs were collected from 480 pigs on 24 pig farms between November 2013 and March 2014. MRSA ST398 was isolated from 54 pigs on five farms. These results indicate that MRSA ST398 has become established in domestic Japanese pigs.

Keywords: MRSA, ST398, pig

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Sasaki Y, Yamanaka M*1, Nara K*1, Tanaka S*1, Uema M, Asai T*2, Tamura Y*3: Isolation of ST398 methicillin-resistant *Staphylococcus aureus* from pigs at abattoirs in Tohoku region, Japan.

Journal of Veterinary Medical Science 2020;82(9):1400-1403. doi: 10.1292/jvms.20-0184

We investigated the presence of ST398 livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) in nasal swabs of 420 slaughtered pigs from 84 farms at three abattoirs in Tohoku, Japan. MRSA were isolated from 13 (3.1%) samples from 9 (10.7%) farms at two abattoirs. All isolates were classified as ST398 and were resistant to ampicillin and tetracycline. Ten and three isolates were classified as Staphylococcal Cassette Chromosome mec (*SCC*mec) types V and IVa, respectively. All type V isolates possessed *czrC*. The minimum inhibitory concentrations (MICs) of zinc chloride against types IVa and V were 1 and 4 mM, respectively. This study shows the presence of ST398 MRSA in pigs in this region. Antimicrobials and zinc compounds in feed and drugs might select *SCC*mec type V ST398 MRSA.

Keywords: MRSA, ST398, pig

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Kumagai Y*1, Pires SM*2, Kubota K, Asakura H: Attributing human foodborne diseases to food sources and water in Japan using analysis of outbreak surveillance data.
Journal of Food Protection 2020;83:2087-2094. doi:10.4315/JFP-20-151

In Japan, strategies for ensuring food safety have been developed without reliable scientific evidence on the relationship between foodborne diseases and food sources. This study aimed to provide information on the proportions of foodborne diseases caused by seven major causative pathogens (*Campylobacter* spp., *Salmonella*, enterohemorrhagic *Escherichia coli* (EHEC), *Vibrio parahaemolyticus, Clostridium perfringens, Staphylococcus aureus*, and Norovirus) attributed to foods and to explore factors affecting changes in these source attribution proportions over time using analysis of outbreak surveillance data. For the calculation of the number of outbreaks attributed to each source, simple-food outbreaks were assigned to the single-food category in question, and complex-food outbreaks were classified under each category proportional to the estimated probability. During 2007 to 2018, 8,730 outbreaks of foodborne diseases caused by seven pathogens were reported, of which 6,690 (76.6%) were of unknown source. We estimated the following source attribution proportions of foodborne diseases: chicken products (80.3%, 95% uncertainty interval [UI] 80.1 to 80.4) for *Campylobacter* spp.; beef products (50.1%, UI 47.0 to 51.5) and vegetables (42.3%, UI 40.9 to 45.5) for EHEC; eggs (34.6%, UI 27.8 to 41.4) and vegetables (34.4%, UI 27.8 to 40.8) for *Salmonella*; finfish (50.3%, UI 33.3 to 66.7) and shellfish (49.7%, UI 33.3 to 66.7) for *V. parahaemolyticus*; grains and beans (57.8%, UI 49.7 to 64.9) for *S. aureus*; vegetables (63.6%, UI 48.5 to 74.6), chicken products (12.7%, UI 4.6 to 21.5), and beef products (11.1%, UI 8.5 to 13.1) for *C. perfringens*; and shellfish (75.5%, UI 74.7 to 76.2) for Norovirus. In this study, we provide the best available evidence-based information to evaluate the link between foodborne diseases and foods. Our results on source attribution for *Campylobacter* spp. and EHEC suggest that the strict health regulations for raw beef were reflected in the proportions of these diseases attributed to this food.
Keywords: foodborne outbreak, foodborne surveillance, source attribution

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Sasaki Y, Sakurada H*1, Yamanaka M*1, Nara K*1, Tanaka S*1, Uema M, Ishii Y*2, Tamura Y*3, Asai T*4: Effectiveness of ear skin swabs for monitoring methicillin-resistant *Staphylococcus aureus* ST398 in pigs at abattoirs.
*Journal of Veterinary Medical Scienc*e 2021;83(1):112-115. doi: 10.1292/jvms.20-0592

To optimize sampling for LA-MRSA monitoring, we compared the sensitivity of MRSA isolation from skin swabs taken behind the ear and nasal swabs collected from 276 pigs and investigated the prevalence of MRSA in their carcasses. MRSA was isolated from 40 behind the ear skin swabs (14.5%), which was statistically higher than the number isolated from nasal swabs (23 samples, 8.3%). MRSA prevalence in the carcasses was 0.4%. All MRSA isolates were sequence type 398 lineage. Sampling of both the skin behind the ear and nasal mucosa in a pig is recommended to investigate the prevalence of LA-MRSA in pigs.
Keywords: MRSA, ST398, pig

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Kawase J*1, Hirai S*2, Yokoyama E*3, Hayashi F*1, Kurosaki M*1, Kawakami Y*1, Fukuma A*1, Sakai T*1, Kotani M*1, Asakura H: Phylogeny, prevalence, and shiga toxin (Stx) production of clinical *Escherichia coli* O157 clade 2 strains isolated in Shimane Prefecture, Japan.
Current Microbiology 2021;78(1):265-273. doi:10.1007/s00284-020-02252-4

This study investigated the genetic and pathogenic variation of the subgroups of clade 2 strains of Shiga toxin (Stx)-producing *Escherichia coli* (STEC) O157. A total of 111 strains of STEC O157 isolated in

Shimane prefecture, Japan, were classified in clade 2 (n = 39), clade 3 (n = 16), clade 4/5 (n = 3), clade 7 (n = 14), clade 8 (n = 17), and clade 12 (n = 22) by single-nucleotide polymorphism analysis and lineage-specific polymorphism assay. These results showed a distinct difference from our previous study in which clade 3 strains were the most prevalent strains in three other prefectures in Japan, indicating that the clade distribution of O157 strains was different in different geographic areas in Japan. Phylogenetic analysis using insertion sequence (IS) 629 distribution data showed that clade 2 strains formed two clusters, designated 2a and 2b. Stx2 production by cluster 2b strains was significantly higher than by cluster 2a strains ($P < 0.01$). In addition, population genetic analysis of the clade 2 strains showed significant linkage disequilibrium in the IS629 distribution of the strains in clusters 2a and 2b ($P < 0.05$). The ΦPT values calculated using the IS629 distribution data indicated that strains in clusters 2a and 2b were genetically different ($P < 0.001$). Cluster 2b strains are a highly pathogenic phylogenetic group and their geographic spread may be a serious public health concern.

Keywords: STEC, clade 2, phylogeny

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Oshiro N, Tomikawa T, Kuniyoshi K, Ishikawa A*1, Toyofuku H*2, Kojima T*3, Asakura H: LC–MS/MS Analysis of Ciguatoxins Revealing the Regional and Species Distinction of Fish in the Tropical Western Pacific.

J Marine Sci Eng. 2021;9(3):299. doi:10.3390/jmse9030299

Ciguatera fish poisoning (CFP) is one of the most frequently reported seafood poisoning diseases. It is endemic to the tropical region and occurs most commonly in the regions around the Pacific Ocean, Indian Ocean, and Caribbean Sea. The principal toxins causing CFP are ciguatoxins (CTXs). In the Pacific region, more than 20 analogs of CTXs have been identified to date. Based on their skeletal structures, they are classified into CTX1B-type and CTX3C-type toxins. We have previously reported species-specific and regional-specific toxin profiles. In this study, the levels and profiles of CTXs in fish present in the tropical western Pacific regions were analyzed using the liquid chromatography–tandem mass spectrometry (LC–MS/MS) technique. Forty-two fish specimens, belonging to the categories of snappers, groupers, Spanish mackerel, and moray eel, were purchased from various places such as Fiji, the Philippines, Thailand, and Taiwan. Only the fish captured from Fijian coastal waters contained detectable amounts of CTXs. The toxin levels in the fish species found along the coastal regions of the Viti Levu Island, the main island in Fiji, and the toxin profiles were significantly different from those of the fish species present in other coastal regions. The toxin levels and profiles varied among the different fish samples collected from different coastal areas. Based on the toxin levels and toxin profiles, the coast was demarcated into three zones. In Zone-1, which covers the northern coast of the main island and the regions of the Malake Island and Korovau, CTXs in fish were below the detection level. In Zone-2, CTX3C-type toxins were present in low levels in the fish. CTX1B-type and CTX3C-type toxins co-occurred in the fish present in Zone-3. The toxin profiles may have reflected the variation in *Gambierdiscus* spp.

Keywords: ciguatera, ciguatoxin, Fiji

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Oshiro N, Nagasawa H, Kuniyoshi K, Kobayashi N*1, Sugita-Konishi Y*1, Asakura H, Yasumoto T*2: Characteristic distribution of ciguatoxins in the edible parts of a grouper, *Variola louti*.

Toxins 2021;13(3):218. doi:10.3390/toxins13030218

Ciguatera fish poisoning (CFP) is one of the most frequently encountered seafood poisoning syndromes; it is caused by the consumption of marine finfish contaminated with ciguatoxins (CTXs). The majority of CFP cases result from eating fish flesh, but a traditional belief exists among people that the head and viscera are more toxic and should be avoided. Unlike the viscera, scientific data to support the legendary high toxicity of the head is scarce. We prepared tissue samples from the fillet, head, and eyes taken from five yellow-edged lyretail (*Variola louti*) individuals

sourced from Okinawa, Japan, and analyzed the CTXs by LC-MS/MS. Three CTXs, namely, CTX1B, 52-*epi*-54-deoxyCTX1B, and 54-deoxyCTX1B, were confirmed in similar proportions. The toxins were distributed nearly evenly in the flesh, prepared separately from the fillet and head. Within the same individual specimen, the flesh in the fillet and the flesh from the head, tested separately, had the same level and composition of toxins. We, therefore, conclude that flesh samples for LC-MS/MS analysis can be taken from any part of the body. However, the tissue surrounding the eyeball displayed CTX levels two to four times higher than those of the flesh. The present study is the first to provide scientific data demonstrating the high toxicity of the eyes.

Keywords: ciguatera, ciguatoxin, LC-MS/MS

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Fukatsu S*1, Horinouchi H*1, Nagata S*1, Kamei R*1, Tanaka D*1, Hong W*1, Kazami Y*1, Fujimori M*1, Itoh K*2, Momose Y, Kasakura K*1, Hosono A*1, Kaminogawa S*1, Hanazawa S*1, Nakanishi Y*1, Takahashi K*1: Post-translational suppression of the high affinity IgE receptor expression on mast cells by an intestinal bacterium.
Immunobiology 2021;226(2):152056. doi:10.1016/j.imbio.2021.152056

Mast cells, which express the high-affinity IgE receptor (Fc ε RI) on their surface, play a crucial role in inducing allergic inflammation. Since mast cells are activated by crosslinking of Fc ε RI with IgE and allergens, the cell surface expression level of Fc ε RI is an important factor in determining the sensitivity to allergens. In this study, it is indicated that *B. acidifaciens* type A43 suppresses cell surface expression of Fc ε RI on mast cells in a post-translational manner via inhibition of Erk. The suppression of Fc ε RI expression on mast cells by specific bacteria might be the underlying mechanism involved in the regulation of allergy by gut microbiota.

Keywords: *Bacteroides acidifaciens*, Fc ε RI, mast cell

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佐々木貴正，岩田剛敏*，上間匡，朝倉宏：牛胆嚢内胆汁のカンピロバクター汚染状況と分離株の性状.
食品衛生学雑誌 2020;61(4):126-131. doi:10.3358/shokueishi.61.126

と畜場において，胆汁のカンピロバクター汚染状況およびその分離株の性状を調査した．カンピロバクターは35.7%（55/154）から分離され，*C. jejuni*と*C. fetus*が上位２菌種であった．*C. jejuni*では，テトラサイクリン（63.0%）とシプロフロキサシン（44.4%）に高率な耐性が認められた．Multi-locus sequence typingにより，*C. jejuni*は12型に分類され，ST806が最も多く，37.0%を占めていた．*C. fetus*では，シプロフロキサシン（66.6%），ストレプトマイシン（58.3%）およびテトラサイクリン（33.3%）に高率な耐性が認められた．すべての*C. fetus*は，ST３（16株）およびST６（８株）に分類された．16株のST３のうち，15株（93.8%）はストレプトマイシンとシプロフロキサシンの両方に耐性であった．と畜場における牛肝臓の胆汁汚染防止は，カンピロバクター感染のリスク低減策の１つである．

Keywords：カンピロバクター，牛胆汁，薬剤耐性

* 農研機構動物衛生部門

佐々木貴正，百瀬愛佳，朝倉宏，浅井鉄夫*：孵化場におけるセフチオフル使用中止後のブロイラー鶏群由来および鶏肉由来サルモネラの薬剤耐性.
鶏病研究会報 2020;56(2):47-52.

孵化場における第３世代セファロスポリン（TGC）使用を2012年３月末に中止した鶏肉生産者１社の協力の下，孵化場，肉用鶏農場および鶏肉のサルモネラ汚染および薬剤耐性の動向調査を実施した．孵化場で採取した種卵80ロットの死ごもり卵検体のうち，12検体（15.0%）からサルモネラが分離されたが，いずれもTGCの１つであるセフォタキシム（CTX）に感受性であった．また，食鳥処理場で採取した24鶏群の盲腸内容物のうち，23鶏群（95.8%）からサルモネラが分離されたが，いずれもCTXに感受性であっ．さらに，鶏肉では，24鶏群のすべてからサルモネラが分離されたが，いずれもCTX感受性であった．孵化場におけるTGC使用中止は，孵化場，肉用鶏農場および鶏肉に由来するサルモネラ株のTGC耐性率低下に貢献したと考えられた．

Keywords：サルモネラ，孵化場，薬剤耐性

* 岐阜大学

佐々木貴正，上間匡，百瀬愛佳，米満研三，浅井鉄夫*，朝倉宏：２食鳥処理場におけるブロイラー群お

よび胸肉のカンピロバクターおよびサルモネラ汚染状況と薬剤耐性.
鶏病研究会報 2020;56(4):153-158.

２カ所の食鳥処理場（中規模および大規模）の協力の下，13鶏群の盲腸内容物とその胸肉を採取し，カンピロバクターとサルモネラの汚染状況とその分離株の薬剤耐性状況を調査した．カンピロバクターは，12鶏群（92.3%）の盲腸内容物と11鶏群（84.6%）の胸肉から分離された．胸肉の汚染菌数については，大規模食鳥処理場（平均2.19 $\log_{10}$ CFU/g）の方が中規模食鳥処理場（平均1.46 $\log_{10}$ CFU/g）と比べ有意に高かった．盲腸内容物または胸肉から分離された*Campylobacter jejuni*の58.3%または36.4%がシプロフロキサシンに耐性であった．サルモネラは調査した全鶏群の盲腸内容物と胸肉から分離された．中規模食鳥処理場では，第３世代セファロスポリンの１つであるセフォタキシムに耐性な*Salmonella* Infantisが５鶏群の盲腸内容物から分離され，その鶏群から加工された胸肉からも分離された．残りの１鶏群の盲腸内容物と胸肉からは*S.* Anatumが分離された．大規模食鳥処理場では，*S.* Schwarzengrundまたは*S.* Manhattanが全鶏群の盲腸内容物と胸肉から分離された．両血清型の大部分がストレプトマイシンとテトラサイクリンの両方に耐性であったが，セフォタキシム耐性は認められなかった.
Keywords：サルモネラ，カンピロバクター，薬剤耐性

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大城直雅，富川拓海，國吉杏子，木村圭介*1，小島尚*2，安元健*3，朝倉宏：卸売市場に搬入された魚類から検出されたシガトキシン類.
食品衛生学雑誌 2021;62(1):8-13. doi: 10.3358/shokueishi.62.8

世界最大規模の自然毒食中毒シガテラの未然防止のために，地方自治体では可能性のある魚種を指定して販売自粛を指導している．水産卸売市場で販売が自粛された魚類７種18試料についてLC-MS/MSによりシガトキシン類（CTXs）を分析した結果，５試料（バラフエダイ４試料およびバラハタ１試料）からCTXsが検出された．含量の高かった２試料（No. 5 : 0.348 μg/kg, No. 8 : 0.362 μg/kg）は200 g程度の摂食で発症すると推定され，販売自粛がCFPを未然に防止したことが示唆された．産地不明のバラフエダイ（１試料）からはCTX1B系列（CTX1B, 52-*epi*-54-deoxyCTX1Bおよび54-deoxyCTX1B）のみが検出され，沖縄・奄美産バラフエダイと組成が類似していることから沖縄・奄美海域で漁獲された可能性が示唆される．一方，和歌山産バラフエダイ（２試料）からはCTX1B系列とCTX3C系列（2,3-dihydroxyCTX3C, 2,3,51-trihydroxyCTX3C, 2-hydroxyCTX3C）の両方が検出された．なお，本州沿岸産魚類からCTXsを検出したのは初めての例である.
Keywords：シガトキシン，バラフエダイ，バラハタ

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Aoki W*, Watanabe M, Watanabe M*, Kobayashi N*, Terajima J, Sugita-Konishi1 Y*, Kondo K, Hara-Kudo Y: Discrimination between edible and poisonous mushrooms among Japanese *Entoloma sarcopum* and related-species based on phylogenetic analysis and insertion/deletion patterns of nucleotide sequences of cytochrome oxidase 1 gene. *Genes and Genetic Systems*. 2020;95:133-139
https://doi.org/10.1266/ggs.19-00032

Entoloma sarcopum is widely known as an edible mushroom but appears morphologically similar to the poisonous mushrooms *E. rhodopolium* sensu lato (s. l.) and *E. sinuatum* s. l. Many cases of food poisoning caused by eating these poisonous mushrooms occur each year in Japan. Therefore, they were recently reclassified based on both morphological and molecular characteristics as sensu stricto species. In this study, we analyzed the nucleotide sequences of the rRNA gene (rDNA) cluster region, mainly including the internal transcribed spacer regions and mitochondrial cytochrome oxidase 1 (CO1) gene, in *E. sarcopum* and its related species, to evaluate performances of these genes as genetic markers for identification and molecular phylogenetic analysis. We found that the CO1 gene contained lineage-specific insertion/deletion sequences, and our CO1 tree yielded phylogenetic information that was not supported by analysis of the rDNA cluster region sequence. Our results suggested that the CO1 gene is a better genetic marker than the rDNA cluster region, which is the most widely used marker for fungal identification and classification, for discrimination between edible and poisonous mushrooms among Japanese *E. sarcopum* and related species. Our study thus reports a new genetic marker that is useful for detection of Japanese poisonous mushrooms, *Entoloma*.
Keywords: CO1 gene, *Entoloma rhodopolium*,

Entoloma sarcopum

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* Azabu University

Arai S, Yoshinari T, Terajima J, Hara-Kudo Y, Ohnishi T: Detection of *Kudoa hexapunctata* and *Kudoa neothunni* from retail raw tuna in Japan using a novel duplex polymerase chain reaction. *Parasitology International*. 2020;75:102048. doi: 10.1016/j.parint.2019.102048

Kudoa hexapunctata was taxonomically separated from *Kudoa neothunni*, but their main host is tuna. *K. hex- apunctata* has been identified as causative agent of foodborne diseases associated with the ingestion of raw Pacific bluefin tuna (PBT) in Japan, but *K. neothunni* has not. Therefore, it is clinically and epidemiologically important to detect and distinguish these two species. In the present study, we developed a novel duplex polymerase chain reaction (dPCR) targeting the 28S rRNA gene sequences of *K. hexapunctata* and *K. neothunni*. The dPCR amplified the desired genetic regions of each species, and the detection limit was 10 copies/reaction. A total of 36 retail tuna samples from different fishing ports were purchased and tested by dPCR. Thirty-one tested positive for *K. hexapunctata* and four tested positive for *K. neothunni*. Several retail PBT samples were examined in some of the fishing ports, and among these samples, the detection rates of *K. hexapunctata* was higher than 85%, and the rates were similar between wild and farmed PBT. The detection rates of *K. hexapunctata* in wild and farmed retail PBT were 75% and 71%, respectively, in May. However, the rates in June and July were 100% for both. *K. hexapunctata* and *K. neothunni* myxospores were not observed in the dPCR-positive samples, except in juvenile PBT, suggesting that the number of parasites was insufficient to cause foodborne disease. Thus, dPCR is a useful method for detecting and distinguishing *K. hexapunctata* and *K. neothunni*, and can be used in epide- miological studies of these parasites.

Keywords: *Kudoa hexapunctata*, *Kudoa neothunni*, duplex PCR, tuna

Bryła M[*1], Ksieniewicz-Woźniak E[*1], Agnieszka Waśkiewicz A[*2], Yoshinari T, Szymczyk K[*1], Podolska G[*3], Gwiazdowski R[*4], Kubiak K[*4]: Transformations of Selected Fusarium Toxins and Their Modified Forms During Malt Loaf Production. *Toxins* (*Basel*). 2020;12:385. doi:10.3390/toxins12060385

An increasing number of studies have found that modified mycotoxins, such as free mycotoxins, naturally occur in food, and severely impact food safety. The present study investigated concentrations of trichothecenes nivalenol, deoxynivalenol, and zearalenone, together with their modified forms, nivalenol-3-glucoside, deoxynivalenol-3-glucoside, and zearalenone-14-glucoside and zearalenone-14-sulfate, respectively, at successive stages of malt loaf production (flour, dough kneading/fermentation, loaf baking). Toxins in bakery products originate in flour produced from wheat grain that is naturally contaminated with *Fusarium culmorum*. Mycotoxin concentrations were determined using high-performance liquid chromatography-high resolution mass spectrometry, and did not significantly change during the successive stages of bread production.

Keywords: Fusarium toxins, baking, bread, malt, modified mycotoxins

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Daud N*, Currie V*, Duncan G*, Farquharson F*, Yoshinari T, Louis P*, Gratz S*. Prevalent Human Gut Bacteria Hydrolyse and Metabolise Important Food-Derived Mycotoxins and Masked Mycotoxins. *Toxins* (*Basel*). 2020;12:654. doi:10.3390/toxins12100654

Mycotoxins are important food contaminants that commonly co-occur with modified mycotoxins such as mycotoxin-glucosides in contaminated cereal grains. These masked mycotoxins are less toxic, but their breakdown and release of unconjugated mycotoxins has been shown by mixed gut microbiota of humans and animals. The role of different bacteria in hydrolysing mycotoxin-glucosides is unknown, and this study therefore investigated fourteen strains of

human gut bacteria for their ability to break down masked mycotoxins. Individual bacterial strains were incubated anaerobically with masked mycotoxins, or unconjugated mycotoxins for up to 48 h. Bacterial growth, hydrolysis of mycotoxin-glucosides and further metabolism of mycotoxins were assessed.

Keywords: de-acetylation, gut microbiota, microbiome, mycotoxin-glucosides, release; trichothecenes

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Hara-Kudo Y, Otsuka K[*1], Konishi N[*2], Yoshida T[*3], Iwabuchi K[*4], Hiratsuka T[*5], Nagai Y[*6], Kimata K[*7] Wada H[*8], Yamazaki T[*9], Tsuchiya A[*10], Mori T[*11], Inagaki S[*12], Shiraishi S[*13], Terajima J: An interlaboratory study on the detection methods for enterotoxigenic *Escherichia coli* in vegetables using enterotoxin gene screening and selective agars for ETEC-specific isolation.

International Journal of Food Microbiology. 2020;334:108832. doi:10.1016/j.ijfoodmicro.2020.108832

Enterotoxigenic *Escherichia coli* (ETEC) causes acute diarrhea and is transmitted through contaminated food and water; however, systematic procedures for its specific detection in foods have not been established. To establish an efficient detection method for ETEC in food, an interlaboratory study using ETEC O148 and O159 as representative serogroups was first conducted with 13 participating laboratories. A series of tests including enrichment, real-time PCR assays, plating on selective agars, and concentration by immunomagnetic separation followed by plating onto selective agar (IMS-plating methods) were employed. This study particularly focused on the detection efficiencies of real-time PCR assays for enterotoxin genes (*sth, stp*, and *lt*), IMS-plating methods, and direct plating onto sorbitol MacConkey agar and CHROMagar STEC medium, supplemented with tobramycin, which is a novel modification in the preparation of a selective agar. Cucumber and leek samples inoculated with ETEC O148 and O159, either at 4–7 CFU/25 g (low levels) or at 21–37 CFU/25 g (high levels) were used as samples with uninoculated samples used as controls. At high inoculation levels, the sensitivities of *sth*, *stp*, and *lt* detection, direct-plating, and IMS-plating methods in cucumber inoculated with O148 and in both foods inoculated with O159 were 100%. In leek inoculated with high levels of O148, the sensitivities of *sth*, *stp*, and *lt* detection, direct-plating, and the IMS-plating method were 76.9%, 64.1%, and 74.4%, respectively. At low inoculation levels, the sensitivities of *sth*, *stp*, and *lt* detection, direct plating, and IMS-plating method in cucumber inoculated with O148 and in both foods inoculated with O159 were in the range of 87.2–97.4%. In leek inoculated with low levels of O148, the sensitivities of *sth*, *stp*, and *lt* detection, direct plating, and the IMS-plating method were 59.0%, 33.3%, and 38.5%, respectively. Thus, ETEC in food contaminated with more than 21 CFU/25 g were detected at high rate (over 74%) using real-time PCR assays and IMS-plating onto selective agar. Therefore, screening *sth*, *stp*, and *lt* genes followed by isolation of STEC using the IMS-plating method may be an efficient method for ETEC detection.

Keywords: ETEC, food, interlaboratory study

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Izawa K[*1], Kubosaki A, Kobayashi N[*2], Akiyama Y[*1], Yamazaki A[*3], Hashimoto K[*4], Konuma R[*5], Kamata Y[*6,7], Hara-Kudo Y, Hasegawa K[*8], Ikaga T[*9], Watanabe M. Comprehensive fungal community analysis of house dust using next-generation

sequencing. *International Journal of Environmental Research and Public Health*. 2020;17:5842
https://doi.org/10.3390/ijerph17165842

Fungal community analyses in homes have been attracting attention because fungi are now generally considered to be allergens. Currently, these analyses are generally conducted using the culture method, although fungal communities in households often contain species that are difficult to culture. In contrast, next-generation sequencing (NGS) represents a comprehensive, labor- and time-saving approach that can facilitate species identification. However, the reliability of the NGS method has not been compared to that of the culture method. In this study, in an attempt to demonstrate the reliability of this application, we used the NGS method to target the internal transcribed spacer 1 in the fungal genome, conducted fungal community analyses for 18 house-dust samples and analyzed fungal community structures. The NGS method positively correlated with the culture method regarding the relative abundance of *Aspergillus*, *Penicillium*, *Cladosporium* and yeasts, which represent the major fungal components found in houses. Furthermore, several genera, such as *Malassezia*, could be sensitively detected. Our results imply that the reliability of the NGS method is comparable to that of the culture method and indicates that easily available databases may require modifications, including the removal of registrations that have not been sufficiently classified at the genus level.

Keywords: house dust, fungal community analysis, next-generation sequencing

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Kubosaki A, Kobayashi N*1, Watanabe M, Yoshinari T, Takatori K*2, Kikuchi Y, Hara-Kudo Y, Terajima J, Sugita-Konishi Y*1: A new protocol for detection of *Aspergillus* section *Versicolores* using a high discrimination polymerase. *Biocontrol Science*. 2020; 25:113-118
https://doi.org/10.4265/bio.25.113

Aspergillus section *Versicolores* species, except *Aspergillus sydowii*, produce a carcinogenic mycotoxin sterigmatocystin (STC). Since these fungi are found in varied environmental milieu including indoor dust and food products, our aim was to develop a sensitive and convenient assay to detect STC producing fungal strains. We made use of a high discrimination DNA polymerase (HiDi DNA polymerase), for single nucleotide polymorphism (SNP)-based PCR amplification. Using specific primer pairs based on the SNPs between A. *sydowii* and other strains of the genomic DNA all target strains except A. *sydowii*. These results confirm that the SNP-based PCR amplification technique, using a high discrimination DNA polymerase, was a reliable and robust screening method for target fungal strains.

Keywords: *Aspergillus* section *Versicolores*, SNP-based PCR amplification, high discrimination polymerase

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Ohnishi T, Furuya A*, Arai S, Yoshinari T, Goto K*, Hara-Kudo Y: Discovery of putative anti-*Kudoa septempunctata* chemical agents by comprehensive assay.
Food Hygiene and Safety Science. 2020;61:183-185. doi:10.3358/shokueishi.61.183.

We screened 360 chemicals and discovered that 71 chemicals had anti-*Kudoa septempunctata* effect. Especially 19 and seven of 71 chemicals were antibiotics and antibacterial agents/disinfectants, respectively. The other 45 chemicals were pesticides, natural toxins, industrial chemicals and medicines for non-infectious diseases. Nineteen antibiotics that possessed anti-*Kudoa* effect contained four tetracyclines, one steroid, two macrolides, one aminoglycoside, three β-lactams, one quinolone, two rifamycines, one polyene, one novobiocine, one sulfonamide and two nitroimidazoles. To use these drugs for prevention of *Kudoa* infection, the further study is need for the determination of effective dose.

Keywords: Kudoa, food-borne disease

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Oshikata C*1,2, Watanabe M, Ishida M*3, Kobayashi S*3, Kubosaki A, Yamazaki A*4, Konuma R*5, Hashimoto K*6, Kobayashi N*7, Kaneko T*2, Kamata Y*8, Yanai M*3, Tsurikisawa N*1,2: Increase in asthma prevalence in adults in temporary housing after the Great East Japan earthquake. *International Journal of Disaster Risk Reduction*. 2020;50:101696
https://doi.org/10.1016/j.ijdrr.2020.101696

It is unknown whether disasters such as earthquakes and tsunamis affect asthma development or exacerbation in adults. Here, we investigated whether asthma prevalence increased in those aged ≧15 years living in temporary housing after the Great East Japan Earthquake. We diagnosed asthma according to GINA guidelines in residents aged ≧15 years who were living, or had lived, in temporary housing in the city of Ishinomaki. We analyzed serum antigen-specific anti-immunoglobulin E (IgE) antibody levels to *Dermatophagoides farinae* (Der f), *Aspergillus fumigatus*, *Aspergillus glaucus*, *Aspergillus amstelodami*, and *Aspergillus restrictus*. The average age of the 337 inhabitants was 61.3±15.8 years (men, 37.7%). The asthma prevalence was 24.9% according to respiratory specialist diagnosis. The antigen-specific IgE antibody titer against Der f, but none of the other test antigens, was significantly higher in the asthma group than in the no-asthma group ($p<0.01$). Time of asthma onset was before the earthquake in 44.6%; in shelters, 9.5%, and after moving into temporary housing, 45.9%. In 71.4% of asthmatics there was exacerbation of asthma after temporary housing occupancy. Logistic regression revealed that the risk factors for developing asthma after moving into temporary housing were allergic rhinitis or allergic conjunctivitis ($p<0.05$), family asthma history ($p<0.05$), never having smoked ($p<0.01$), and peripheral airways disorder (low % V50) ($p<0.05$) but not depression. The earthquake and tsunami disasters increased mite allergen sensitization and exacerbation or development of asthma in temporary housing residents aged ≧15 years.

Keywords: adult asthma, Great East Japan Earthquake, temporary housing

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Watanabe M, Konuma R*1, Kobayashi N*2, Yamazaki A*3, Kamata Y*3,4, Hasegawa K*5, Kimura N*6, Tsurikisawa N*7,8, Oshkata C*7,8, Sugita-Konishi Y*2, Takatori K*9, Yoshino H*10, Hara-Kudo Y: Indoor fungal contamination in temporary housing after the East Japan Great Earthquake Disaster. *International Journal of Environmental Research and Public Health*. 2021;18:3296
https://doi.org/10.3390/ijerph18063296

To understand fungal contamination in the indoor environment of the disaster region, a field survey was performed to measure the number of fungal counts and identify isolates in the indoor air of prefabricated temporary housing, privately independent-housing, and rented apartments flooded by the East Japan Great Earthquake disaster tsunami. As a result, the period with the highest detected fungal count was from the rainy season to summer in independent-housing and rented apartments. Moreover, in the temporary housing, the fungal number increased further in winter as indicated by the maximum fungal-number throughout the measurement period. The detection frequency of *Aspergillus* species was relatively higher in the indoor air of temporary housing than in typical housing in the non-disaster area. Since *Aspergillus* is known as an allergenic genus, it requires careful attention to the health risk for residents. The extremely high level of fungal condensation in indoor air possibly occurred due to high relative humidity and loss of heat insulation in the building attics. It is suggested that this problem commonly happened in the cold region including the entire disaster region of the East Japan Great Earthquake.

Keywords: mycoflora, Great East Japan Earthquake, temporary housing, *Aspergillus*

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Yoshinari T, Watanabe M, Ohnishi T, Hara-Kudo Y: Assessment of Modified Forms of Fumonisins in Corn-Based Products Retailed in Japan by an Alkaline Hydrolysis Method.
Food Hygiene and Safety Science. 2020;61:119-125. doi:10.3358/shokueishi.61.119.

Fumonisins, which are secondary metabolites produced by some *Fusarium* species, are detected mainly in corn and corn-based products. Recently, the presence of modified forms of fumonisins in fumonisin-contaminated food products has been reported. In order to evaluate the health risk of modified forms of fumonisins to the Japanese population, we analyzed modified forms of fumonisins in corn-based products retailed in Japan. The modified and free forms of fumonisins in food samples were hydrolyzed by alkaline treatment. The resulting hydrolyzed fumonisins were quantified by LC-MS/MS, and total fumonisins (sum of modified and free forms) was calculated. A total of 166 samples of corn-based products were analyzed over two years.
Keywords: corn-based products, fumonisin, modified mycotoxin

湯之前雄太*1，林克彦，大谷梓*2，松村佳代子*3，中尾亮介*3，毛利聡里*3，古田美玲，小原有弘*2，河合充生*3，内田恵理子，清水則夫*1，伊豆津健一，工藤由起子，菊池裕：マイコプラズマ否定試験に用いるマイコプラズマ参照品に関する研究（第２報）第十七改正日本薬局方収載NBRC由来マイコプラズマの核酸増幅法（NAT）による検出感度に関する共同比較研究.
医薬品医療機器レギュラトリーサイエンス
2020;51:224-233

第十七改正日本薬局方参考情報のマイコプラズマ否定試験では，核酸増幅法（NAT）を利用する場合には，用いる手法が，十分な検出感度を持つことを，バリデーションで示す必要がある。マイコプラズマ参照品は，ゲノムコピー（GC）数及びコロニー形成数（CFU数）が測定された凍結菌株であり，ゲノムの核酸配列を検出するNATにおいて，バリデーションの陽性対照として使用される。製品評価技術基盤機構バイオテクノロジーセンター（NBRC）から分譲されるマイコプラズマ菌株では，陽性対照としての妥当性検証が不十分であったことから，再検証を目的として，参考情報に収載されたNBRC由来のマイコプラズマ５菌株及びアコレプラズマ１菌株，また，参考情報に未収載の*Mycoplasma arginini* NBRC 111899の計７菌種に，試験の対照として，参考情報に収載され十分な検証がなされた*M. arginini* 23838を加えた合計８菌株を培養して，参照品を調製し，市販されるNATキット４種で試験した。全ての菌株で，検出感度が10 CFU/mL未満であり，十分な感度で検出されたことから，これらの菌株は，NATのバリデーションの陽性対照として妥当であることが示された.
Keywords: mycoplasma testing, nucleic acid amplification test (NAT), validation

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Hirano M, Saito C*, Yokoo H, Goto C, Kawano R*, Misawa T, Demizu Y: Structure-activity relationship analysis of Mag2-based peptide foldamers.
Molecules, 2021, 26, 444. doi: 10.3390/molecules26020444

Magainin 2 (Mag 2), which is isolated from the skin of frogs, is a representative antimicrobial peptide (AMP), exerts its antimicrobial activity via microbial membrane disruption. It has been reported that both the amphipathicity and helical structure of Mag 2 play an important role in its antimicrobial activity. In this study, we revealed that the sequence of 17 amino acid residues in Mag 2 (peptide 7) is required to exert sufficient activity. We also designed a set of Mag 2 derivatives, based on enhancement of helicity and/or amphipathicity, by incorporation of α,α-disubstituted amino acid residues into the Mag 2 fragment, and evaluated their preferred secondary structures and their antimicrobial activities against both Gram-positive and Gram-negative bacteria. As a result, peptide 11 formed a stable helical structure in

solution, and possessed potent antimicrobial activities against both Gram-positive and Gram-negative bacteria without significant cytotoxicity.
Keywords: antimicrobial peptides; magainin 2; stapled peptide; helical structure; amphipathicity

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Yuyama M*1, Misawa T, Demizu Y, Kanaya T*1, Kurihara M*1: Design and synthesis of novel estrogen receptor antagonists with acetal containing biphenylmethane skeleton.
Results in Chemistry, 2021, 3, 100124. doi: 10.1016/j.rechem.2021.100124

The estrogen receptor (ER) is a member of the nuclear receptor family, wherein ERα is a subtype of the estrogen receptor. The ERα receptor is primarily expressed in the mammary glands and the gonads, and is associated with human breast cancer. Several studies have reported that approximately 75% of human breast cancer cases are hormone-dependent (ERα-positive). Therefore, several ERα antagonists, such as tamoxifen, are frequently used to treat ERα-positive breast cancer (Fig. 1). As reported, the biphenylmethane skeleton has been used as a template for developing nuclear receptor ligands, such as those for the ER and vitamin D receptors (VDR), since this skeleton is known to stably bind to these receptors. In previous studies, various types of ERα antagonists bearing the biphenylmethane skeleton have been developed and reported to exhibit good ERα antagonistic activities. For example, Maruyama et al. reported that bisphenol A analogs (BPA analogs) exhibited strong ERα inhibitory activities (Fig. 1). It has been suggested that the hydrophobic moiety in the center of the biphenylmethane unit is characteristic of a structure that exhibits a good ERα antagonist activity. These BPA analogs were synthesized from ketones and phenols in low yields (7–35%).
Keywords: ER antagonist; Acetal; Biphenylmethane skeleton

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Terui R, Yanase Y, Yokoo H, Suhara Y*1, Makishima M*2, Demizu Y, Misawa T: Development of novel selective TGR5 ligands based on 5,6,7,8-tetrahydro-5,5,8,8- tetramethylnaphtalene skeleton.
ChemMedChem, 2021, 16, 458-462. doi: 10.1002/cmdc.202000567

TGR5, a G-protein-coupled receptor (GPCR), plays an important role in several physiological functions. TGR5 activation through bile acids induces an increase in energy expenditure. Therefore, synthetic TGR5 ligands could be useful for the treatment of obesity or dyslipidemia. In this study, we designed and synthesized a set of TGR5 ligands with a 5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene (TMN) skeleton, and evaluated their TGR5 agonistic activity. We also investigated the selectivity of the synthesized compounds for TGR5 relative to the farnesoid X receptor (FXR) and retinoic acid receptor (RAR). Our results show that compound 4b [N-(2-chlorophenyl)-5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenecarboxamide] exhibited potent TGR5 agonist activity with an IC50 value of 8.4 nM without significant cytotoxicity. In addition, compound 4b showed only slight agonistic activity toward FXR and RAR at 1μM treatment. These data indicate that compound 4b is a selective TGR5 agonist, and could be a promising therapeutic agent for dyslipidemia.
Keywords: GPCR, TGR5, Bile acids, Retinoid, FXR

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Hirano M, Saito C*, Goto C, Yokoo H, Kawano R*, Misawa T, Demizu Y: Rational design of helix-stabilized antimicrobial peptide foldamers containing α,α-disubstituted amino acids or side-chain stapling.
ChemPlusChem, 2020, 85, 2731-2736. doi: 10.1002/cplu.202000749

Antimicrobial peptides (AMPs) are expected to be good candidate molecules for novel antimicrobial therapies. Most AMPs exert their antimicrobial activity through disruption of microbial membranes due to their amphipathic properties. Recently, the helical peptide 'Stripe' was reported by our group, a rationally designed amphipathic AMP focused on distribution of natural cationic and hydrophobic amino acid residues. In this study, a set of Stripe-based AMP foldamers was designed, synthesized and investigated that contain α,α-disubstituted amino acids or side-

chain stapling to stabilize their helical structures. Our results showed that a peptide containing 2-aminoisobutyric acid (Aib) residues exhibited potent antimicrobial activity against both Gram-positive S.aureus (MIC value: 3.125 μM) and Gram-negative bacteria (including a multidrug-resistant strain, MDRP, MIC value: 1.56 μM), without significant hemolytic activity (>100 μM). Electrophysiological measurements revealed that this peptide formed stable pores in a 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) /1,2-dioleoyl-sn-glycero-3-phosphoglycerol (DOPG) bilayer but not in a dioleoylphosphocholine (DOPC) bilayer. The introduction of Aib residues into Stripe could be a promising way to increase the antimicrobial activity of AMP foldamers, and the peptide could represent a promising novel therapeutic candidate to treat multidrug-resistant bacterial infection.

Keywords: amphipathic peptides; antimicrobial activity, foldamers, helical structures, hemolysis

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Ikeda K, Yanase Y, Hayashi K, Hara-Kudo Y, Tsuji G, Demizu Y: Amine skeleton-based c-di-GMP derivatives as biofilm formation inhibitors.

Bioorg. Med. Chem.Lett. 2020, 32, 127713. doi: 10.1016/j.bmcl.2020.127713

Bacteria can form a biofilm composed of diverse bacterial microorganism, which work as a barrier to protect from threats, such as antibiotics and host immunity system. The formation of biofilms significantly impairs the efficacy of antibiotics against pathogenic bacteria. It is also a serious problem to be solved that the emergence of multidrug-resistant bacteria (such as methicillin-resistant Staphylococcus aureus, MRSA) accelerated by the overuse of antibiotics. Therefore, the usage of biofilm inhibition agents has attracted immense interest as a novel strategy for treatment of diseases related to bacterial infection. From the difference of mode of action against bacterial cells, biofilm inhibition agents are expected to circumvent the emergence of multidrug-resistant bacteria. In this study, we have developed the derivatives of c-di-GMP, a kind of cyclic dinucleotide that is expected to have the effect of inhibiting bacterial biofilm formation. Some of the synthesized derivatives were found to inhibit biofilm formation of Gram-positive bacteria.

Keywords: Amine skeleton, Biofilm formation inhibitor, Cyclic dinucleotide, Gram-negative bacteria, Gram-positive bacteria, c-di-GMP

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Sakuraba S*1, Iwakiri J*2, Hamada M*3, Kameda T*4, Tsuji G, Kimura Y*5, Abe H*5, Asai K*2: Free-Energy Calculation of Ribonucleic Inosines and Its Application to Nearest-Neighbor Parameters.

J. Chem. Theory Comput. 2020, 16, 5923-5935. doi: 10.1021/acs.jctc.0c00270

Can current simulations quantitatively predict the stability of ribonucleic acids (RNAs)? In this research, we apply a free-energy perturbation simulation of RNAs containing inosine, a modified ribonucleic base, to the derivation of RNA nearest-neighbor parameters. A parameter set derived solely from 30 simulations was used to predict the free-energy difference of the RNA duplex with a mean unbiased error of 0.70 kcal/mol, which is a level of accuracy comparable to that obtained with parameters derived from 25 experiments. We further show that the error can be lowered to 0.60 kcal/mol by combining the simulation-derived free-energy differences with experimentally measured differences. This protocol can be used as a versatile method for deriving nearest-neighbor parameters of RNAs with various modified bases.

Keywords: Free energy, Melting, Genetics, Chemical calculations, Nucleic acid structure

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Nakamoto K*1, Abe N*1, Tsuji G, Kimura Y*1, Tomoike F*1, Shimizu Y*2, Abe H*1: Chemically synthesized circular RNAs with phosphoramidate linkages enable rolling circle translation.

Chem. Commn., 2020, 56, 6217-6220. doi: 10.1039/d0cc02140g

Circular RNA without a stop codon enables rolling circle translation. To produce circular RNAs, we carried out one-pot chemical synthesis of circular RNA from RNA fragments with the use of an EDC/HOBt-based chemical ligation reaction. The synthesized circular RNAs acted as translation templates, despite the presence of unnatural phosphoramidate linkages.

Keywords: circular RNA, chemical ligation, cyclization, phosphoramidate

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Mizuno M[*], Mori K[*], Tsuchiya K, Misawa T, Demizu Y, Shibanuma M[*], Fukuhara K[*]: Design, synthesis, and biological activity of conformationally-restricted analogues of silibinin.

ACS Omega, 2020, 5, 23164-23174. doi: 10.1021/acsomega.0c02936

Silibinin (Sib), one of the main components of milk thistle extract, has attracted considerable attention because of its various biological activities, which include antioxidant activity and potential effects in diabetes and Alzheimer's disease (AD). In a previous study, we synthesized catechin analogues by constraining the geometries of (+)-catechin and (−)-epicatechin. The constrained analogues exhibited enhanced bioactivities, with the only major difference between the two being their three-dimensional structures. The constrained geometry in (+)-catechin resulted in a high degree of planarity (PCat), while (−)-epicatechin failed to maintain planarity (PEC). The three-dimensional structure of Sib may be related to its ability to inhibit aggregation of amyloid beta (Aβ). We therefore introduced PCat and PEC into Sib to demonstrate how the constrained molecular geometry and differences in three-dimensional structures may enhance such activities. Introduction of PCat into Sib (SibC) resulted in effective inhibition of Aβ aggregation, α-glucosidase activity, and cell growth, suggesting that not only reduced flexibility but also the high degree of planarity may enhance the biological activity. SibC is expected to be a promising lead compound for the treatment of several diseases.

Keywords: Peptides and proteins, Chemical structure, Aggregation, Inhibition, Toxicity

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Abe Y, Yamamoto E, Yoshida H, Usui A, Tomita N, Kanno H, Masada S, Yokoo H, Tsuji G, Uchiyama N, Hakamatsuka T, Demizu Y, Izutsu K, Goda Y, Okuda H: Formation of *N*-nitrosodimethylamine (NDMA) from ranitidine powders and tablets under high-temperature storage conditions.

Chem. Pharm. Bull., 2020, 68, 1008-1012. doi: 10.1248/cpb.c20-00431

The purpose of this study was to elucidate the effect of high-temperature storage on the stability of ranitidine, specifically with respect to the potential formation of *N*-nitrosodimethylamine (NDMA), which is classified as a probable human carcinogen. Commercially available ranitidine reagent powders and for- mulations were stored under various conditions, and subjected to LC-MS/MS analysis. When ranitidine tablets from two different brands (designated as tablet A and tablet B) were stored under accelerated condi- tion (40℃ with 75% relative humidity), following the drug stability guidelines issued by the International Conference on Harmonisation (ICH-Q1A), for up to 8 weeks, the amount of NDMA in them substantially increased from 0.19 to 116 ppm and from 2.89 to 18 ppm, respectively. The formation of NDMA that exceeded the acceptable daily intake limit (0.32 ppm) at the temperature used under accelerated storage conditions clearly highlights the risk of NDMA formation in ranitidine formulations when extrapolated to storage under ambient conditions. A forced-degradation study under the stress condition (60℃ for 1 week) strongly suggested that environmental factors such as moisture and oxygen are involved in the formation of NDMA in ranitidine formulations. Storage of ranitidine tablets and reagent powders at the high temperatures also increased the amount of nitrite, which is considered one of the factors influencing NDMA formation. These data indicate the necessity of controlling/monitoring stability-related factors, in addition to controlling impurities during the manufacturing process, in order to mitigate nitrosamine-related health risks of certain pharmaceuticals.

Keywords: *N*-nitrosodimethylamine (NDMA),

ranitidine, forced degradation, storage; impurity

Ueda A[*1], Ikeda M[*1], Kasae T[*1], Doi M[*2], Oba M[*1], Demizu Y, Tanaka M[*1]: Synthesis of chiral alpha-trifluoromethyl alpha,alpha-disubstituted alpha-amino acids and conformational analysis of L-Leu-based peptides having an (*R*)- or a (*S*)-alpha-trifluoromethylalanine.

ChemistrySelect, 2020, 5, 10882-10886. doi: 10.1002/slct.202002888

Various racemic alpha-trifluoromethyl alpha,alpha-disubstituted alpha-amino acids were synthesized by the reaction of methyl 3,3,3-trifluoropyruvate imines with Grignard reagents. The optical resolution of racemates using (*R*)-1,1'-bi-2-naphthol {(*R*)-BINOL} esters gave optically active alpha-trifluoromethylated alpha,alpha-disubstituted alpha-amino acids, such as alpha-trifluoromethylalanine (alpha-CF_3Ala), alpha-trifluoromethylleucine (alpha-CF_3Leu), and alpha- trifluoromethylphenylalanine (alpha-CF_3Phe). The optically active (*R*)- or (*S*)-alpha-CF_3Ala was incorporated into the L-Leu-based pentapeptides, and their preferred conformation in solution and in the crystal state was studied by Fourier transform infrared (FT-IR) absorption, nuclear Overhauser effect spectroscopy (NOESY) NMR, and circular dichroism (CD) spectra, as well as X-ray crystallographic analysis. Both L-Leu-based peptides with (*R*)- or (*S*)-alpha-CF_3Ala formed right-handed 3_{10}-helical structures. Both peptide-backbones at the N-terminal residues 1-3 were very similar, but the φ and ψ torsion angles of residues 4 and 5 between peptides with (*R*)- or (*S*)-alpha-CF_3Ala were different.

Keywords: amino acid, conformation, helix・peptide, trifluoromethyl

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Tsuji G, Yusa M, Masada S, Yokoo H, Hosoe J, Hakamatsuka T, Demizu Y, Uchiyama N: Facile synthesis of kwakhurin, a marker compound of *Pueraria mirifica*, and its quantitative NMR analysis for standardization as a reagent.

Chem. Pharm. Bull., 2020, 68, 797-801. doi: 10.1248/cpb.c20-00346

The side effects of kwao keur dietary supplements (obtained from the tuberous root of Pueraria mirifica) have recently been reported by the Ministry of Health, Labour and Welfare, Japan. To control the quality of kwao keur products, its ingredients need to be maintained by characteristic marker compounds, such as miroestrol, deoxymiroestrol, and kwakhurin (KWA). In this study, we described the facile synthesis of KWA, a marker compound of P. mirifica. Our revised synthetic method produced KWA with shorter steps and higher yield than the reported method. Furthermore, the absolute purity of KWA was determined by quantitative NMR analysis for standardization as a reagent, and its purity was 92.62 ± 0.12%.

Keywords: Kwakhurin, *Pueraria mirifica*, synthesis, quantitative NMR

Yokoo H, Ohoka N, Naito M, Demizu Y: Design and synthesis of peptide-based chimeric molecules to induce degradation of the estrogen and androgen receptors.

Bioorg. Med. Chem., 2020, 28, 115595. doi: 10.1016/j.bmc.2020.115595

Peptide-based inducers of estrogen receptor (ER) α and androgen receptor (AR) degradations via the ubiqui- tin–proteasome system (UPS) were developed. The designated inducers were composed of two biologically active scaffolds: the helical peptide PERM3, which is an LXXLL-like mimic of the coactivator SRC-1, and various small molecules (MV1, LCL161, VH032, and POM) that bind to E3 ligases (IAPs, VHL, and cereblon, respectively), to induce ubiquitylation of nuclear receptors that bind to SRC-1. All of the synthesized chimeric E3 ligand-containing molecules induced the UPS-mediated degradation of ERα and AR. The PERM3 peptide was applicable for the development of the ERα and AR degraders using these E3 ligands.

Keywords: Nuclear receptors; Helical peptide; Protein–protein interaction; Protein knockdown

Yamamoto K*, Tsuda Y*, Kuriyama M*, Demizu Y, Onomura O*: Cu-catalyzed enantioselective synthesis of oxazolines from aminotriols via asymmetric desymmetrization.

Chem. Asian J., 2020, 15, 840-844. doi: 10.1002/asia.201901742

A copper-catalyzed enantioselective transformation of tris(hydroxymethyl)aminomethane-derived aminotriols was developed to provide multisubstituted oxazolines with a tetrasubstituted carbon center. The present transformation consisted of sequential reactions involving mono-sulfonylation of aminotriols, subsequent intramolecular cyclization to afford prochiral oxazoline diols, and sulfonylative asymmetric desymmetrization of resultant oxazoline diols. In addition, the kinetic resolution process would be involved in the sulfonylative asymmetric desymmetrization step, which would amplify the enantiopurities of the desired products. Various aminotriols were tolerated in the present reaction, affording the desired oxazolines in good to high yields with excellent enantioselectivities. The synthetic utility of the present reaction was demonstrated by the transformation of the optically active oxazoline into a chiral α-tertiary amine motif.

Keywords: copper, asymmetric synthesis, α-tertiary amines, triols, oxazolines

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Yamano K[*1], Kikuchi R[*1,2], Kojima W[*1,2], Hayashida R[*1], Koyano F[*1], Kawawaki J[*1], Shoda T, Demizu Y, Naito M, Tanaka K[*1], Matsuda N[*1]: Essential roles of ubiquitin signals and OPTN-ATG9A axis in mitochondria selective autophagy.

J. Cell. Biol., 2020, 219, e201912144. doi: 10.1083/jcb.201912144

Damaged mitochondria are selectively eliminated in a process called mitophagy. Parkin and PINK1, proteins mutated in Parkinson's disease, amplify ubiquitin signals on damaged mitochondria with the subsequent activation of autophagic machinery. Autophagy adaptors are thought to link ubiquitinated mitochondria and autophagy through ATG8 protein binding. Here, we establish methods for inducing mitophagy by mitochondria-targeted ubiquitin chains and chemical-induced mitochondrial ubiquitination. Using these tools, we reveal that the ubiquitin signal is sufficient for mitophagy and that PINK1 and Parkin are unnecessary for autophagy activation per se. Furthermore, using phase-separated fluorescent foci, we show that the critical autophagy adaptor OPTN forms a complex with ATG9A vesicles. Disruption of OPTN–ATG9A interactions does not induce mitophagy. Therefore, in addition to binding ATG8 proteins, the critical autophagy adaptors also bind the autophagy core units that contribute to the formation of multivalent interactions in the de novo synthesis of autophagosomal membranes near ubiquitinated mitochondria.

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Shibata N, Ohoka N, Tsuji G, Demizu Y, Miyazawa K[*1], Ui-Tei K[*2], Akiyama T[*2], Naito M: Deubiquitylase USP25 prevents degradation of BCR-ABL protein and ensures proliferation of Ph-positive leukemia cells.

Oncogene, 2020, 39, 3867-3878. doi: 10.1038/s41388-020-1253-0

Fusion genes resulting from chromosomal rearrangements are frequently found in a variety of cancer cells. Some of these are known to be driver oncogenes, such as BCR-ABL in chronic myelogenous leukemia (CML). The products of such fusion genes are abnormal proteins that are ordinarily degraded in cells by a mechanism known as protein quality control. This suggests that the degradation of BCR-ABL protein is suppressed in CML cells to ensure their proliferative activity. Here, we show that ubiquitin-specific protease 25 (USP25) suppresses the degradation of BCR-ABL protein in cells harboring Philadelphia chromosome (Ph). USP25 was found proximal to BCR-ABL protein in cells. Depletion of USP25 using shRNA-mediated gene silencing increased the ubiquitylated BCR-ABL, and reduced the level of BCR-ABL protein. Accordingly, BCR-ABL-mediated signaling and cell proliferation were suppressed in BCR-ABL-positive leukemia cells by the depletion of USP25. We further found that pharmacological inhibition of USP25 induced rapid degradation of BCR- ABL protein in Ph-positive leukemia cells, regardless of their sensitivity to tyrosine kinase inhibitors. These results indicate that USP25 is a novel target for inducing the degradation of oncogenic BCR-ABL protein in Ph-positive leukemia cells. This could be an effective approach to overcome resistance to kinase inhibitors.

Keywords: Deubiquitylase, USP25, BCR-ABL, chronic myelogenous leukemia

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Ishimoto H*1, Kano M*2, Sugiyama H*3, Takeuchi H*4, Terada K*5, Aoyama A*6, Shoda T, Demizu Y, Shimamura J*8, Yokoyama R*9, Miyamoto Y*10, Hasegawa K*11, Serizawa M*12, Unosawa K*13, Osaki K*14, Asai N*15, Matsuda Y*16: Approach to Establishment of Control Strategy for Oral Solid Dosage Forms Using Continuous Manufacturing.
Chem Pharm Bull. 2021, 69(2), 211-217. doi: 10.1248/cpb.c20-00824

As a result of the research activities of the Japan Agency for Medical Research and Development (AMED), this document aims to show an approach to establishing control strategy for continuous manufacturing of oral solid dosage forms. The methods of drug development, technology transfer, process control, and quality control used in the current commercial batch manufacturing would be effective also in continuous manufacturing, while there are differences in the process development using continuous manufacturing and batch manufacturing. This document introduces an example of the way of thinking for establishing a control strategy for continuous manufacturing processes.
Keywords: Quality by Design (QbD), continuous manufacturing, control strategy, regulatory science, solid drug product, state of control

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Takabatake R*1, Ohnishi M*2, Minegishi Y*3, Futo S*2, Soga K, Nakamura K, Kondo K, Mano J*1, Kitta K*1: Development of a Novel Detection Method Targeting an Ultrashort 25 bp Sequence Found in Agrobacterium-Mediated GM Plants.
J. Agric. Food Chem. 2020;68:15327-15334. DOI: 10.1021/acs.jafc.0c03864

Agrobacterium-mediated transformation is the most commonly used technique for plant genetic engineering. During the transformation, a T-DNA region, which is flanked by the right border (RB) and the left border, is transferred to plant nuclear chromosomes. Simultaneously, a sequence adjacent to the RB on T-DNA is frequently transferred to plant genomes together with the intentionally introduced recombinant DNA. We developed a novel polymerase chain reaction (PCR)-mediated detection method targeting this region. The conserved sequence of the region found in genetically modified (GM) crops is only 25 bp in length. To detect this ultrashort 25 bp sequence near the RB region, we designed a primer set consisting of a 12-base forward primer and a 13-base reverse primer. The predicted band was detected from GM crops by optimizing the PCR conditions. We used lateral flow DNA chromatography for rapid and inexpensive detection. The developed method would be applicable for screening the GM crops generated by *Agrobacterium*-mediated transformation.
Keywords: genetically modified (GM), polymerase chain reaction (PCR), screening detection

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高畠令王奈*，大西真理*，真野潤一*，岸根雅宏*，曽我慶介，中村公亮，近藤一成，橘田和美*：遺伝子組換えトウモロコシ定量のための内標比の算出.
食品衛生学雑誌, 2020;61:235-238. DOI: 10.3358/shokueishi.61.235

安全性審査済み遺伝子組換え（GM）トウモロコシおよびダイズの粉砕試料中の混入率を，重量混合比として算出するためには，内標比が必要である．内標比は，GMダイズに関しては，リアルタイムPCR新機種QuantStudio5, QuantStudio12K Flex, LightCycler 96およびLightCycler 480において，組換え配列と内在性配列のコピー数比を基に既に測定されているが，GMトウモロコシに関しては未対応であった．本研究では，GM

トウモロコシのスクリーニング検査法の対象であるカリフラワーモザイクウイルス35Sプロモーター，GA21構造特異的領域，MIR604系統特異的領域，MIR162系統特異的領域において，上記リアルタイムPCR 4 機種を用いて内標比を算出した.

Keywords：遺伝子組換え，リアルタイムPCR，内標比

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* 農研機構

Soga K, Kimata S, Narushima J, Sato S[*1], Sato E[*1], Mano J[*2], Takabatake R[*2], Kitta K[*2], Kawakami H[*1], Akiyama H, Kondo K, Nakamura K: Development and Testing of an Individual Kernel Detection System for Genetically Modified Soybean Events in Non-identity-preserved Soybean Samples.

Biol. Pharm. Bull. 2020;43:1259-1266. DOI: 10.1248/bpb.b20-00382

A genetically modified (GM) soybean kernel detection system using combination of DNA preparation from individual soybean kernels and event-specific real-time PCR was developed to simultaneously identify GM soybean events authorized for food after safety assessments in Japan. Over 100 kernels in the non-identity-preserved soybean samples imported from the United States of America (two U.S.A. lots) and Brazil (one lot) were randomly selected and examined. In total, 98 and 96% of the two independent U.S.A. lots, and 100% of the Brazilian lot contained GM soybean kernels. Herbicide-tolerant events, MON89788 (trade name Genuity® Roundup Ready 2 Yield™), GTS 40-3-2 (trade name Roundup Ready™ soybean) and A2704-12 (trade name Liberty Link® soybean), were detected similarly in both U.S.A. lots. In the Brazilian lot, in addition to GTS 40-3-2, a stacked GM event, MON87701 × MON89788, having insect-resistance and herbicide-tolerance, was detected. There were no unauthorized GM soybeans comingled, and the ratio of GM soybean events detected was consistent with statistical reports on the cultivated GM soybean events in both countries.

Keywords: event identification, genetically modified (GM), real-time PCR

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Shibata N, Ohoka N, Tsuji G, Demizu Y, Miyawaza K[*1], Ui-Tei K[*2], Akiyama T[*2], Naito M[*2]: Deubiquitylation USP25 prevents degradation of BCR-ABL protein and ensures proliferation of Ph-positive leukemia cells.

Oncogene. 2020;39:3867-3878. DOI: 10.1038/s41388-020-1253-0.

Fusion genes resulting from chromosomal rearrangements are frequently found in a variety of cancer cells. Some of these are known to be driver oncogenes, such as BCR-ABL in chronic myelogenous leukemia (CML). The products of such fusion genes are abnormal proteins that are ordinarily degraded in cells by a mechanism known as protein quality control. This suggests that the degradation of BCR-ABL protein is suppressed in CML cells to ensure their proliferative activity. Here, we show that ubiquitin-specific protease 25 (USP25) suppresses the degradation of BCR-ABL protein in cells harboring Philadelphia chromosome (Ph). USP25 was found proximal to BCR-ABL protein in cells. Depletion of USP25 using shRNA-mediated gene silencing increased the ubiquitylated BCR-ABL, and reduced the level of BCR-ABL protein. Accordingly, BCR-ABL-mediated signaling and cell proliferation were suppressed in BCR-ABL-positive leukemia cells by the depletion of USP25. We further found that pharmacological inhibition of USP25 induced rapid degradation of BCR-ABL protein in Ph-positive leukemia cells, regardless of their sensitivity to tyrosine kinase inhibitors. These results indicate that USP25 is a novel target for inducing the degradation of oncogenic BCR-ABL protein in Ph-positive leukemia cells. This could be an effective approach to overcome resistance to kinase inhibitors.

Keywords: chronic myelogenous leukemia (CML), deubiquitylation, degradation

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Narushima J, Kimata S, Soga K, Sugano Y[*1], Kishine M[*2], Takabatake R[*2], Mano J[*2], Kitta K[*2], Kanamaru S[*3], Shirakawa N[*3], Kondo K, Nakamura K: Rapid DNA template preparation directly from a rice sample without purification for loop-mediated isothermal amplification (LAMP) of rice genes.

Biosci Biotechnol Biochem. 2020;84:670-677. DOI: 10.1080/09168451.2019.1701406

Rapid DNA template preparation directly from a

single rice (*Oryza sativa*) grain or rice flour of its equivalent weight was developed for loop-mediated isothermal amplification (LAMP). LAMP efficiency using DNA extract obtained from consecutive addition of alkaline lysis reagent (25 mM NaOH, 0.2 mM EDTA) and neutralizing reagent (40 mM Tris-HCl [pH 5]) was comparable to that using an equivalent amount of purified DNA as template. The stability of the prepared DNA extract was confirmed for up to six-day storage at room temperature. Without using any special laboratory devices, the developed method enabled a rapid, simple, and low-cost DNA template preparation method for reliable LAMP testing to detect rice genes.

Keywords: Loop-mediated isothermal amplification; genetically modified; rice grain

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戸渡寛法*1，宮﨑悦子*1，赤木浩一*2，中牟田啓子*1，片岡洋平，渡邉敬浩：新規開発したLC-MS/MS法を用いた魚に含まれる有機ヒ素化合物の分析．
食品衛生学雑誌 2021;61:86-94 https://doi.org/10.3358/shokueishi.61.86

多くの魚に複数の種類の有機ヒ素化合物が含まれているが，化学形態ごとに毒性が異なることから，長期摂取による健康影響のリスクを評価するためには，形態別に濃度を定量する必要がある．本研究では，魚中のモノメチルアルソン酸（MMA），ジメチルアルシン酸（DMA），トリメチルアルシンオキサイド（TMAO），テトラメチルアルソニウム（TeMA），アルセノベタイン（AB），アルセノコリン（AC）を対象としたLC-MS/MSによる分析法を開発し，妥当性を確認した．また，福岡市内に流通する魚10種（計50試料）について総ヒ素濃度および各有機ヒ素化合物濃度を調査した．その結果，総ヒ素はすべての試料から0.53～25 mg/kgの範囲で検出され，カワハギからは8.3～25 mg/kgの範囲で検出された．イワシを除く9種においては，総ヒ素濃度に占める各化合物濃度のうち，AB濃度の割合が最も高かったが，イワシにおいてはAB濃度よりDMA濃度の割合が高く，総ヒ素濃度のうち16～24%を占めていた．養殖マダイにおける総ヒ素，ABおよびACの濃度は天然マダイより低かった．

Keywords：有機ヒ素化合物，実態調査，魚

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Watanabe T, Matsuda R, Uneyama C: Probabilistic Estimation of Dietary Intake of Methylmercury from Fish in Japan.
Food Safety. 2021;9:1-9 https://doi.org/10.14252/foodsafetyfscj.D-20-00018

Dietary intake of methylmercury from fish was estimated via Monte Carlo simulation using data for methylmercury concentrations in 210 fish samples and data regarding fish consumption extracted from the Japanese National Health and Nutrition Survey. The fish analyzed were classified into 5 groups according to categories used in the survey. The distribution of consumption of fish from each group was used without fitting to statistical distributions. A log-normal distribution was fitted to the distribution of methylmercury concentration in each fish group. Two random numbers that followed these distributions were generated, and a trial value was calculated by multiplying these random numbers. The trial value was divided by the body weight (50 kg) to arrive at an estimate of dietary methylmercury intake. A total of 100,000 Monte Carlo simulation iterations were performed. The estimated mean daily intake of methylmercury was 0.093 μg/kg body weight (bw)/day. This value is well below the tolerable daily intake of 0.292 μg/kg bw/day calculated from the tolerable weekly intake (2.0 μg/kg bw/week) established by the Food Safety Commission of Japan. The probability that the daily intake of methylmercury exceeds the tolerable daily intake was 7.6%. As there are no data regarding fish consumption for consecutive days, estimation of the weekly intake of methylmercury is a subject for future studies.

Keywords: Methylmercury, Dietary intake, Probabilistic estimation

Yuko Kumagai*1, Sara Monteiro Pires*2, Kunihiro Kubota, Hiroshi Asakura: Attributing Human Foodborne Diseases to Food Sources and Water in Japan Using Analysis of Outbreak Surveillance Data.
Journal of Food Protection. 2020 Dec 1;83(12):2087-2094

In Japan, strategies for ensuring food safety have been developed without reliable scientific evidence

on the relationship between foodborne diseases and food sources. This study aimed to provide information on the proportions of foodborne diseases caused by seven major causative pathogens (Campylobacter spp., Salmonella, enterohemorrhagic Escherichia coli [EHEC], Vibrio parahaemolyticus, Clostridium perfringens, Staphylococcus aureus, and norovirus) attributed to foods and to explore factors affecting changes in these source attribution proportions over time using analysis of outbreak surveillance data. For the calculation of the number of outbreaks attributed to each source, simple-food outbreaks were assigned to the single-food category in question, and complex-food outbreaks were classified under each category proportional to the estimated probability. During 2007 to 2018, 8,730 outbreaks of foodborne diseases caused by seven pathogens were reported, of which 6,690 (76.6%) were of unknown source. We estimated the following source attribution proportions of foodborne diseases: chicken products (80.3%, 95% uncertainty interval [UI] 80.1 to 80.4) for Campylobacter spp.; beef products (50.1%, UI 47.0 to 51.5) and vegetables (42.3%, UI 40.9 to 45.5) for EHEC; eggs (34.6%, UI 27.8 to 41.4) and vegetables (34.4%, UI 27.8 to 40.8) for Salmonella; finfish (50.3%, UI 33.3 to 66.7) and shellfish (49.7%, UI 33.3 to 66.7) for V. parahaemolyticus; grains and beans (57.8%, UI 49.7 to 64.9) for S. aureus; vegetables (63.6%, UI 48.5 to 74.6), chicken products (12.7%, UI 4.6 to 21.5), and beef products (11.1%, UI 8.5 to 13.1) for C. perfringens; and shellfish (75.5%, UI 74.7 to 76.2) for norovirus. In this study, we provide the best available evidence-based information to evaluate the link between foodborne diseases and foods. Our results on source attribution for Campylobacter spp. and EHEC suggest that the strict health regulations for raw beef were reflected in the proportions of these diseases attributed to this food.

Keywords: Foodborne outbreaks, Foodborne surveillance, Source attribution

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Akiyama H*1, Kawamata K*2, Fukutomi Y*3, Matsufuji H*2, Kai S*4, Miyazawa M*4, Nakamura R: Novel *in vitro* test for pollen-related vegetable/fruit allergy using the EXiLE method.

Allergology International. 2020;69:459-461. doi: 10.1016/j.alit.2019.12.007.

Pollen-associated food allergy syndrome (PFS), which is a common problem worldwide, develops when an individual who is sensitized to an inhaled pollen ingests fruits or vegetables that cross-react with the sensitizing pollen allergen. The *in vivo* prick-prick test using fresh fruits or vegetables is the most sensitive test for detecting IgE to food in PFS. However, test results vary depending on the overall condition of the patient, and the number of antigens that can be tested at one time is limited. We established a novel allergy test based on IgE crosslinking-induced luciferase expression (EXiLE) using a humanized cultured rat mast cell line, which expresses human FcεRI and the nuclear factor of activated T-cell-responsive luciferase reporter gene. Using preserved sera, preserved extracts, and a cultured mast cell line, the EXiLE method can detect cross-reactive IgE and allergen-specific IgE from several microliters of serum. The use of freshly prepared tomato or apple skin juice, which is a stimulating antigen, was also effective. Therefore, the EXiLE method can be used for the screening of potentially cross-reactive fruit and vegetable allergens in patients allergic to pollen. In addition to pollen allergen-specific IgE titers, this novel *in vitro* method may help clinicians in diagnosing PFS before performing the *in vivo* prick-prick test, which is burdensome.

Keywords: Pollen-associated food allergy syndrome, EXiLE method, prick-prick test

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Sun Y, Nitta S*1, Saito K, Hosogai R*1, Nakai K*1, Goda R*2, Kakehi M*3, Murata K*4, Yamaguchi T*4, Okuzono T*5, Yamane S*5, Enoki Y*6, Kawabata M*6, Takahara K*7, Sato S*8, Yoshida T, Inoue T, Saito Y: Development of a bioanalytical method for an antisense therapeutic using high-resolution mass spectrometry.

Bioanalysis. 2020;12:1739-1756. doi:10.4155/bio-2020-0225.

Ion-pairing reverse-phase LC coupled with high-resolution mass spectrometry (IP-LC/HRMS) has gained attention in oligonucleotide therapeutic bioanalyses owing to its high sensitivity and selectivity. However, optimization and validation of IP-LC/HRMS-based methods are rare. The objective of this study is the development of a sensitive and reproducible IP-LC/HRMS-based bioanalytical method using clinically approved mipomersen as a model for antisense oligonucleotides.

Mipomersen was extracted from rat plasma using Clarity OTX SPE and quantified by IP-LC/HRMS. The calibration range was 0.5-250.0 ng/ml. The developed method met the general regulatory criteria for accuracy, precision, carry-over, selectivity, matrix effect and dilution integrity. In conclusion, a highly sensitive and reliable method for mipomersen measurement with potential antisense oligonucleotide bioanalysis applications has been developed.

Keywords: antisense oligonucleotide, clarity OTX, bioanalysis

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Imatoh T, Sai K, Saito Y: The association between concurrence of infection and the onset of severe eruption or liver injury in patients using antipyretic analgesics: A matched, nested case-control study.
J Clin Pharmacol. 2020;60:1177-1184. doi: 10.1002/jcph.1613

Stevens-Johnson syndrome (SJS) and toxic epidermal necrosis (TEN) or drug-induced liver injury (DILI) are severe drug-induced reactions, known as idiosyncratic drug reactions. It is believed that immune response can lead to these severe adverse drug reactions. We conducted a matched, nested case-control study to elucidate the association between concurrent infection and the onset of SJS/TEN or liver injury in patients prescribed antipyretic analgesics. We extracted 4,112,055 patients who were prescribed antipyretic analgesics between January 2014 and December 2015. Amongst them, 553 (0.01%) were diagnosed with SJS/TEN and 12,606 (0.3%) with liver injury. In a matched, nested case-control study, 131 and 2847 cases matched for SJS/TEN or liver injury, respectively. For each case, 3 controls were randomly matched with the case for age at index date and sex. In the conditional logistic regression analysis, there was a significant association between the combination of infection and antipyretic analgesics and the onset of SJS/TEN or liver injury (SJS/TEN: adjusted OR, 5.59; 95%CI, 2.01-15.51; liver injury: adjusted OR, 2.79; 95%CI, 2.24-3.46). Although it was not possible to distinguish whether the associations were caused by the infection or were a direct consequence of the antibiotic agents, our findings may help to increase awareness of the possibility of the increased onset of idiosyncratic drug reactions (SJS/TEN and liver injury) in antipyretic analgesic users because of infections.

Keywords: Stevens-Johnson syndrome, toxic epidermal necrosis, drug-induced liver injury

Nakamura R, Ozeki T*1, Hirayama N*2, Sekine A*3, Yamashita T*3, Mashimo Y*3, Mizukawa Y*4, Shiohara T*4, Watanabe H*5, Sueki H*5, Ogawa K*6, Asada H*6, Kaniwa N, Tsukagoshi E, Matsunaga K*7, Niihara H*8, Yamaguchi Y*9, Aihara M*9, Mushiroda T*1, Saito Y, Morita E*8: Association of HLA-A*11:01 with Sulfonamide-Related Severe Cutaneous Adverse Reactions in Japanese Patients.
J Invest Dermatol. 2020;140:1659-1662. doi: 10.1016/j.jid.2019.12.025.

Sulfonamides, globally used as antibacterial agents and anti-rheumatic drugs, can cause Stevens–Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN) and drug-induced hypersensitivity syndrome (DIHS) which are categorized as severe cutaneous adverse reactions (SCARs). In the present study, we examined HLA types associated with SJS/TEN cases from Japanese patients, and found significantly higher frequencies of *HLA-A*11:01* than in the corresponding healthy volunteer population ($P_{corrected} = 0.0034$), and its association was also observed in DIHS cases. Overall odds ratio for SCARs was 9.84 and $P = 2.67 \times 10^{-5}$. In these SCAR cases, influence of slow acetylator genotypes of arylamine N-acetyltransferase 2 (NAT2), a metabolic enzyme of sulfonamides, was only

marginal. Furthermore, we performed *in silico* docking simulations to predict interactions between HLA and sulfonamides, and found that the sulfamethoxazole and salazosulfapyridine were predicted to have affinity to the peptide-binding groove of HLA-A*11:01 with energies of −5.60 and −6.67 kcal/mol, respectively. These values corresponded to 50% inhibitory concentrations of 78 and 13 μM, respectively, which are lower than clinical concentrations of these sulfonamides. Furthermore, Arg114 and Gln156 of HLA-A*11:01 were predicted to be important residues for sulfonamide interactions. These findings suggested *HLA-A*11:01* as a risk factor for sulfonamide-induced onset of SCARs in the Japanese population.

Keywords: Severe cutaneous adverse reactions, Sulfa drugs, HLA-A*11:01

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Hattori N*, Takumi A*, Saito K, Saito Y: Effects of serial cervical or tail blood sampling on toxicity and toxicokinetic evaluation in rats.

J Toxicol Sci. 2020;45:599-609. doi: 10.2131/jts.45.599.

To assess the influences of blood sampling volumes or sites on toxicological and toxicokinetic (TK) evaluations, 4-week duration animal studies and a single-dose TK study of imipramine were conducted. In the toxicological evaluation, six-week-old Sprague-Dawley rats were divided into no blood and blood sampling groups. Fifty microliters (microsampling) or 100 μL (larger sampling) of blood/time point was collected from the jugular vein (50 μL of data was reported previously as Yokoyama et al., 2020) or the tail vein 6 to 7 times on days 1/2 and in week 4. Although no parameters were affected by the 100 μL sample from the tail vein, the 100 μL jugular vein sampling decreased the red blood cell parameters in females, possibly due to hemorrhage at the sampling site. Regarding the TK assessment, 50 μL of blood/site/time point was collected at 6 time points from the tail and jugular vein of the same male rats after single oral administration of 10 or 100 mg/kg imipramine, which was selected as a representative drug with high distribution volume. Although there were no differences in the AUC0-24hr and Cmax values between the sites, the plasma concentrations at the early time points were significantly lower from the tail vein than the jugular vein. From our studies, 50 μL of jugular and tail vein microsampling did not affect the toxicity parameters or AUC/Cmax. However, appropriate toxicity considerations and/or selection of the blood sampling site may be important in the case of larger sampling volumes or blood concentration assessment.

Keywords: Microsampling, Rats, Sampling site

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Saito K, Hattori K*1, Andou T*2, Satomi Y*3, Gotou M*3, Kobayashi H*3, Hidese S*1, Kunugi H*1: Characterization of Postprandial Effects on CSF Metabolomics: A Pilot Study with Parallel Comparison to Plasma.

Metabolites. 2020;10:185. doi: 10.3390/metabo10050185.

Cerebrospinal fluid (CSF) metabolites reflect biochemical diffusion/export from the brain and possibly serve as biomarkers related to brain disease severity, pathophysiology, and therapeutic efficacy/toxicity. Metabolomic studies using blood matrices have demonstrated interindividual and preanalytical variation of blood metabolites, whereas those of CSF metabolites remain unclear. In this study, we aimed to delineate the postprandial effects on CSF metabolites because fasting of patients with brain-related disorders is challenging. We collected pre- and postprandial (1.5, 3, and 6 h) plasma and CSF from nine healthy subjects. Using a mass-spectrometry-based global metabolomics approach, 150 and 130 hydrophilic metabolites and 263 and 340 lipids were detected in CSF and plasma, respectively. Principal component analysis of CSF hydrophilic metabolites and lipids primarily classified individual subjects at any time point, suggesting that the postprandial effects had a lower impact than interindividual variations on CSF metabolites. Individually, less than 10% of the CSF metabolites were putatively altered by postprandial

effects (with either significant differences or over 2-fold changes, but not both) at any time point. Thus, global CSF metabolite levels are not directly associated with food intake, and except for several putatively altered CSF metabolites, postprandial effects are not a major concern when applying CSF metabolomics to screen biomarkers.

Keywords: CSF metabolites, lipidomics, metabolomics

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Saito K, Kagawa T*1, Tsuji K*2, Kumagai Y*3, Sato K*4, Sakisaka S*5, Sakamoto N*6, Aiso M*7, Hirose S*1, Mori N*2, Tanaka R*3, Uraoka T*4, Takata K*5, Ogawa K*6, Mori K*8, Sato M*9, Nishiya T*8, Takamatsu K*9, Arakawa N, Izumi T*10, Ohno Y*10, Saito Y, Takikawa H*7: Plasma Lipid Profiling of Three Types of Drug-Induced Liver Injury in Japanese Patients: A Preliminary Study.

Metabolites. 2020;10:355. doi: 10.3390/metabo10090355.

Drug-induced liver injury (DILI) is a major adverse event caused by drug treatment, which can be categorized into three types: hepatocellular, mixed, and cholestatic. Although nearly every class of drugs can cause DILI, an overall understanding of lipid profiles in DILI patients is lacking. We used lipidomics to analyze the plasma lipid profiles of patients to understand their hepatic pathophysiology and identify DILI biomarkers. We identified 463 lipids and compared their levels between the acute and recovery phases of the three types of DILI patients. Mixed and cholestatic types demonstrated specific plasma lipid alterations between the phases, but the hepatocellular type did not. Moreover, as specific indicators of mixed-type DILI, levels of several ceramides increased in the acute phase, while those of arachidonic acid-containing ether-linked phosphoglycerolipids decreased. In contrast, as specific indicators of cholestatic-type DILI, levels of palmitic acid-containing saturated or monounsaturated phosphatidylcholines increased in the acute phase, while those of arachidonic acid- or docosahexaenoic acid-containing ether-linked phosphoglycerolipids and phosphatidylinositols decreased. We also identified lipids with a relatively high capacity to discriminate the acute phase from the recovery phase and healthy subjects. These findings may help with understanding the pathophysiology of different DILI types and identify candidate biomarkers.

Keywords: biomarker, drug-induced liver injury, lipidomics

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Yokoyama H*1, Hattori N*2, Ohtsuka H*3, Murata E*4, Kobayashi A*1, Muta K*1, Takumi A*2, Kitaura H*3, Jinno F*3, Iwai A*4, Nakai K*4, Mori K*4, Saito K, Saito Y: Lack of toxicological influences by microsampling (50 μL) from jugular vein of rats in a collaborative 28-day study.

J Toxicol Sci. 2020;45:319-325. doi: 10.2131/jts.45.319.

Due to finalization of the ICH S3A Q&A focusing on microsampling, application of microsampling technique to regular non-clinical animal studies is expected for non-clinical safety assessment of pharmaceuticals. In Europe, microsampling from the tail vein or saphenous vein has often been used, whereas sampling from the jugular vein is thought to be more common for non-clinical studies in Japan. Therefore, we assessed the toxicological effects of serial microsampling from the jugular vein of SD rats in a common 28-day study at 4 independent organizations. Fifty microliter sampling was performed at 6 timepoints on day 1 to 2 and 7 timepoints on day 27 to 28 and its toxicological influences on body weight, food consumption, hematological and clinical chemistry parameters, and organ weights (on day 29 for 3 and day 28 for 1 organizations) were evaluated. The serial microsampling was shown to have no or minimal influences on the assessed parameters. The observed statistical differences for the 18 parameters were sporadic and did not appear to be systemically

associated with microsampling. However, the sporadic changes were more often observed in females (14/18 parameters) than in males (6/18), suggesting the possibility that female rats were more susceptible to treatment-based influences. The current results indicate that serial 50 μL sampling from the jugular vein of SD rats had no or very slight toxicological effects, suggesting that this microsampling condition is applicable for toxicokinetic evaluation of non-clinical rat toxicity studies.

Keywords: Microsampling, Rats, Toxicological influence.

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*4 LSI Medience Co.

Maeda M*1, Tanaka R*1, Aso M*2, Sakamoto Y*3, Song I*1, Ochiai M*1, Saito Y, Maekawa K, Arakawa N, Ohno Y*4, Kumagai Y*1: Hepatic Adaptation to Therapeutic Doses of acetaminophen: An Exploratory Study in healthy Individuals.

Clin Ther, 2020;42:1276-1291. doi: 10.1016/j.clinthera.2020.05.003.

Acetaminophen (APAP) has hepatotoxic potential when overdosed. Recent studies have reported serum alanine aminotransferase (ALT) elevations that resolve spontaneously with continued use of the drug, referred to as adaptation, in several individuals receiving therapeutic doses of APAP. However, the clinical significance of these ALT elevations remains unclear. This study was performed to investigate the incidence and characteristics of hepatic adaptation to therapeutic doses of APAP in healthy individuals. In a randomized, single-blind, placebo-controlled study, 242 healthy Japanese individuals were enrolled. Each person received 3 g/d of APAP (n = 202) or placebo (n = 40) for 28 days. All study participants underwent analysis of genetic polymorphisms of CYP2E1 and UGT1A1; measurements of plasma APAP concentration and urine metabolites (glucuronide, sulfate, cysteine, and mercapturate); liver function monitoring, including ALT, microRNA-122, and high-mobility group box 1. Individuals with ALT levels remaining below the upper limit of normal (ULN; 40 U/L) during the study period were defined as tolerant and those with ALT elevations above the ULN as susceptible. Susceptible individuals who developed ALT elevations exceeding 2 × ULN discontinued use of the study drug for tolerability consideration. Susceptible individuals who had ALT elevations that decreased toward the ULN spontaneously with continued use of the study drug were classified as adaptation. In the APAP group, 129 individuals (66%) were classified as tolerant and 65 (34%) as susceptible. Among 65 susceptible individuals, 12 (18%) discontinued use of APAP because of ALT elevations (>2 × ULN), whereas 53 (82%) completed 28-day APAP dosing. Thirty of 65 susceptible individuals (46%) had adaptation within 28 days. In the placebo group, no individuals was withdrawn from the study because of elevated ALT levels, 33 individuals (89%) were classified as tolerant, and 4 (11%) were classified as susceptible. None had clinical signs of liver injury. ALT level correlated significantly with microRNA-122 but not with high-mobility group box 1. No association was found between plasma APAP concentrations and ALT levels. Urinary excretion of APAP mercapturate was higher in susceptible than in tolerant individuals (P = 0.018, Wilcoxon or Kruskal-Wallis test). The frequency of homozygotes and compound heterozygotes for UGT1A1＊28 and UGT1A1＊6 (＊28/＊28, ＊6/＊28, and ＊6/＊28) was higher in susceptible than in tolerant individuals (13.9% vs 3.9%; P = 0.011, $\chi 2$ test). These findings indicate that in healthy individuals, APAP at a therapeutic dose can cause transient and self-limiting ALT elevation, reflecting subclinical hepatocellular damage, and these ALT elevations may be associated with the disposition of APAP metabolites and genetic factors. UMIN-CTR identifier: UMIN000019607.

Keywords: Acetaminophen, Adaptation, Pharmacokinetics.

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Tsuboi I*, T Harada*, Hirabayashi Y, Aizawa S*: Dynamics of hematopoiesis is disrupted by impaired hematopoietic microenvironment in a mouse model of hemophagocytic lymphohistiocytosis.

Ann. Hematol. 2020;99(7):1515-1523. doi 10.1007/s00277-020-04095-2

Hemophagocytic lymphohistiocytosis (HLH) is a life-threatening systemic hyperinflammatory disorder. We found recently that repeated lipopolysaccharide (LPS) treatment induces HLH-like features in senescence-accelerated mice (SAMP1/TA-1) but not in senescence-resistant control mice (SAMR1). In this study, we analyzed the dynamics of hematopoiesis in this mouse model of HLH. When treated repeatedly with LPS, the numbers of myeloid progenitor cells (CFU-GM) and B-lymphoid progenitor cells (CFU-preB) in the bone marrow (BM) rapidly decreased after each treatment in both strains. The number of CFU-GM in SAMP1/TA-1 and SAMR1, and of CFU-preB in SAMR1, returned to pretreatment levels by 7 days after each treatment. However, the recovery in the number of CFU-preB in SAMP1/TA-1 was limited. In both strains, the BM expression of genes encoding positive regulators of myelopoiesis (granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), and interleukin (IL)-6), and negative regulators of B lymphopoiesis (tumor necrosis factor (TNF)-alpha) was increased. The expression of genes encoding positive regulators of B lymphopoiesis (stromal-cell derived factor (SDF)-1, IL-7, and stem cell factor (SCF)) was persistently decreased in SAMP1/TA-1 but not in SAMR1. Expression of the gene encoding p16(INK4a) and the proportion of beta-galactosidase-positive cells were increased in cultured stromal cells obtained from LPS-treated SAMP1/TA-1 but not in those from LPS-treated SAMR1. LPS treatment induced qualitative changes in stromal cells, which comprise the microenvironment supporting appropriate hematopoiesis, in SAMP1/TA-1; these stromal cell changes are inferred to disrupt the dynamics of hematopoiesis. Thus, hematopoietic tissue is one of the organs that suffer life-threatening damage in HLH.

Keywords: LPS, Hemophagocytic lymphohistiocytosis, hematopoietic microenvironment

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Kuwagata M, Muneoka K*, Honda K*, Miyazaki A*: Hypothalamic Monoaminergic Pathology in a Neurodevelopmental Rat Model Showing Prenatal 5-Bromo-2'-Deoxyuridine Treatment-Induced Hyperactivity and Hyporeproductivity.

Neuropsychobiology 2020;79(2):161-169. doi: 10.1159/000504552.

Objective: Prenatal treatment of rats with 5-bromo-2'-deoxyuridine (BrdU) is a neurodevelopmental model showing hyperactivity and impaired sexual activity. Human neurodevelopmental disorders, such as autism, exhibit sex-related pathology, but sex-related neurodevelopment has not been fully investigated in this model. We conducted this study to facilitate the understanding of the pathophysiology of neurodevelopmental disorders.

Methods: Pregnant rats received 50 mg/kg BrdU on gestational days 9-15. The tissue content of dopamine (DA), serotonin (5-HT), and their metabolites dihydroxyphenylacetic acid, homovanillic acid, and 5-hydroxyindoleacetic acid were measured in male and female offspring at 3 weeks (juveniles) and 10 weeks (adults) of age.

Results: Prenatally BrdU-treated rats had reduced DA metabolism or DA content in the hypothalamus from the juvenile through the adult period without sex differences, but sex-specific striatal DA abnormalities emerged after maturation. A reduction in 5-HT metabolism was measured in the hypothalamus without sex differences throughout development. Developmental alterations in the striatal 5-HT states were sex-dependent. Temporal changes in DA or 5-HT metabolism were found in the frontal cortex and midbrain.

Conclusion: The sex-specific influence of a genotoxic factor on the development of the DA and 5-HT systems was clarified in the hypothalamus and striatum. The results suggest that the observed sex dependence and region specificity are related to the pathology of social dysfunction in neurodevelopmental disorders.

Keywords: hyperactivity, hypothalamus, sexual activity

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Kumamoto T*1, Senuma M*2, Todoroki M*2, Kumagai F*2, Imai H*3, Suzuki R*3, Ogawa T*4, Kuwagata M: 5-Fluorocytosine induces fetal skeletal malformations in rats by altering expression of

Homeobox genes.

Fundam. Toxicol. Sci. 2020;7(2):97-103. doi.org/10.2131/fts.7.97

5-Fluorocytosine (5-FC) is an antimycotic and teratogenic compound. Oral administration of 5-FC to pregnant rats on gestation days (GD) 9 and 13 was shown to induce thoracolumbar supernumerary ribs (TSR, 14th rib) and abnormal digits, respectively, in fetuses. This study investigated the effects of 5-FC on homeobox genes, which control the anterior-posterior-axis. 5-FC (75 mg/kg) was administered orally on GD9 and GD13, and tissues collected from cranial and caudal regions of TSR sites were analyzed. Following 5-FC administration on GD9, the levels of expression of Hoxa10, which determine the position of the thoracic and lumbar vertebrae, were decreased at GD13. Analysis of hindlimbs 6 hours after administration on GD13 showed decreases in expression of Hoxa11, Hoxd12, and Hoxd13, the Hox genes responsible for limb formation from the proximal to distal, and from the anterior to posterior directions. The present findings showed that altered expression of Hox genes contributes to 5-FC teratogenicity.

Keywords: 5-FC (flucytosine), homeobox, thoracolumbar supernumerary rib

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Ono R, Yoshioka Y*1, Furukawa Y, Naruse M*2, Kuwagata M, Ochiya T*3, Kitajima S, Hirabayashi Y: Novel hepatotoxicity biomarkers of extracellular vesicle (EV)-associated miRNAs induced by CCl4.

Toxicology Reports 2020;29(7):685-692. doi 10.1016/j.toxrep.2020.05.002

Recent findings have revealed that extracellular vesicles (EVs) are secreted from cells and circulate in the blood. EVs are classified as exosomes (40－100 nm), microvesicles (50－1,000 nm) or apoptotic bodies (500－2,000 nm). EVs contain mRNAs, microRNAs, and DNAs and have the ability to transfer them from cell to cell. Recently, especially in humans, the diagnostic accuracy of tumor cell type-specific EV-associated miRNAs as biomarkers has been found to be more than 90%. In addition, microRNAs contained in EVs in blood are being identified as specific biomarkers of chemical-induced inflammation and organ damage. Therefore, microRNAs contained in the EVs released into the blood from tissues and organs in response to adverse events such as exposure to chemical substances and drugs are expected to be useful as novel biomarkers for toxicity assessment. In this study, C57BL/6 J male mice orally dosed with carbon tetrachloride (CCl4) were used as a hepatotoxicity animal model. Here, we report that not only the known hepatotoxicity biomarkers miR-122 and miR-192 but also 42 novel EV-associated biomarkers were upregulated in mice dosed with CCl4. Some of these novel biomarkers may be expected to be able to use for better understanding the mechanism of toxicity. These results suggest that our newly developed protocol using EV-associated miRNAs as a biomarker would accelerate the rapid evaluation of toxicity caused by chemical substances and/or drugs.

Keywords: exosome, biomarker, carbon tetrachloride (CCl4)

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Saito H, Hara K*, Kitajima S, Tanemura K*: Effect of vitamin E deficiency on spermatogenesis in mice and its similarity to aging.

Reprod. Toxicol. 2020;98:225-232. doi: 10.1016/j.reprotox.2020.10.003.

Vitamin E (VE) plays numerous important roles in mammals because of its antioxidant activity. As a result, VE deficiency (VED) leads to the dysfunction of central nervous, reproductive, and immune systems. However, few studies have reported the effects of VED on the male reproductive system. In this study, we investigated the effects of VED on male reproductive function and examined its relationship to involution in the male reproductive system with aging. We fed a VED or control diet to 4-week-old mice for 12 or 24 weeks. Following the histopathological analysis of

reproductive organs, we found seminiferous tubules with exfoliation in the VED groups, and its frequency was significantly increased compared with the controls. Additionally, in the epididymis, a decrease in spermatozoa and an increase in apoptotic germ cells were observed in the VED groups compared with the controls. By Papanicolaou staining, we also found an increase in the proportion of sperm with abnormal morphology in the VED groups compared with the controls. These reproductive effects induced by VED were highly similar to one aspect of those observed in aged mice. Our findings demonstrate that the aging of the male reproductive system may be accelerated because of the impaired in vivo antioxidant capacity induced by VED.

Keywords: testis, vitamin E, spermatogenesis

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Nock R*1, Polouliakh N*2, Nielsen F*2, Oka K*3, Connell C R*4, Heimhofer C*5, Shibanai K*6, Ghosh S*7, Aisaki KI, Kitajima S, Kanno J, Akama T*2, Kitano H*2,7: A Geometric Clustering Tool (AGCT) to robustly unravel the inner cluster structures of time-series gene expressions.

PLoS One 2020;15(7):e0233755. doi 10.1371/journal.pone.0233755

Systems biology aims at holistically understanding the complexity of biological systems. In particular, nowadays with the broad availability of gene expression measurements, systems biology challenges the deciphering of the genetic cell machinery from them. In order to help researchers, reverse engineer the genetic cell machinery from these noisy datasets, interactive exploratory clustering methods, pipelines and gene clustering tools have to be specifically developed. Prior methods/tools for time series data, however, do not have the following four major ingredients in analytic and methodological view point: (i) principled time-series feature extraction methods, (ii) variety of manifold learning methods for capturing high-level view of the dataset, (iii) high-end automatic structure extraction, and (iv) friendliness to the biological user community. With a view to meet the requirements, we present AGCT (A Geometric Clustering Tool), a software package used to unravel the complex architecture of large-scale, non-necessarily synchronized time-series gene expression data. AGCT capture signals on exhaustive wavelet expansions of the data, which are then embedded on a low-dimensional non-linear map using manifold learning algorithms, where geometric proximity captures potential interactions. Post-processing techniques, including hard and soft information geometric clustering algorithms, facilitate the summarizing of the complete map as a smaller number of principal factors which can then be formally identified using embedded statistical inference techniques. Three-dimension interactive visualization and scenario recording over the processing helps to reproduce data analysis results without additional time. Analysis of the whole-cell Yeast Metabolic Cycle (YMC) moreover, Yeast Cell Cycle (YCC) datasets demonstrate AGCT's ability to accurately dissect all stages of metabolism and the cell cycle progression, independently of the time course and the number of patterns related to the signal. Analysis of Pentachlorophenol iduced dataset demonstrat how AGCT dissects data to identify two networks: Interferon signaling and NRF2-signaling networks.

Keywords: gene expression clustering, software, system biology

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Takaya M*, Matsuda R*, Inamori G*, Kamoto U*, Isoda Y*, Tachibana D*, Nakamura F*, Fuchiwaki O*, Okubo Y, Ota H*: Transformable Electrocardiograph Using Robust Liquid-Solid Heteroconnector.

ACS Sens. 2021;6(1):212-219. doi:10.1021/acssensors.0c02135

In this study, a highly transformable electrocardiograph that can considerably deform the position of stretchable electrodes based on the lead method for diagnosing heart disease was developed; these electrodes exhibited high resistance stability

against considerable stretching and multiple stretching. To realize the large deformable functionality of the electrodes of a system, liquid metal electrodes and a heteroconnector composed of a liquid metal paste and carbon-based conductive rubber were employed. The developed device can achieve a 200% strain with only 6% resistance change and a high stability of resistances after the 100-time stretching test. In addition, the study demonstrated electrocardiograms in different lead methods of adult and child using the same device. The proposed combination of large deformable electrodes with high electric stability and a robust heteroconnector is an important technology, and it presents a considerable advancement in the application of stretchable electronic systems.

Keywords: wearable ECG, liquid metal, electric connector

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Marx-Stoelting P[*1], Solano MLM[*1], Aoyama H[*2], Adams RH [*3], Bal-Price A[*4], Buschmann J[*5], Chahoud I[*6], Clark R[*7], Fang T[*8], Fujiwara M[*9], Gelinsky M[*10], Grote K[*6], Horimoto M[*11], Bennekou SH[*12], Kellner R[*13], Kuwagata M, Leist M[*14], Lang A[*6], Li W[*8], Mantovani A[*15], Makris SL[*16], Paumgartten F[*17], Perron M[*18], Sachana M[*19], Schmitt A[*1], Schneider S[*20], Schönfelder G[*21], Schulze F[*1], Shiota K[*22], Solecki R[*1]: 25th anniversary of the Berlin workshop on developmental toxicology: DevTox database update, challenges in risk assessment of developmental neurotoxicity and alternative methodologies in bone development and growth.

Reprod. Toxicol. 2021;100:155-162. doi: 10.1016/j.reprotox.2020.11.003

25 years after the first Berlin Workshop on Developmental Toxicity this 10th Berlin Workshop aimed to bring together international experts from authorities, academia and industry to consider scientific, methodologic and regulatory aspects in risk assessment of developmental toxicity and to debate alternative strategies in testing developmental effects in the future. Proposals for improvement of the categorization of developmental effects were discussed as well as the update of the DevTox database as valuable tool for harmonization. The development of adverse outcome pathways relevant to developmental neurotoxicity (DNT) was debated as a fundamental improvement to guide the screening and testing for DNT using alternatives to animal methods. A further focus was the implementation of an in vitro mechanism-based battery, which can support various regulatory applications associated with the assessment of chemicals and mixtures. More interdisciplinary and translation research should be initiated to accelerate the development of new technologies to test developmental toxicity. Technologies in the pipeline are (i) high throughput imaging techniques, (ii) models for DNT screening tests, (iii) use of computer tomography for assessment of thoracolumbar supernumerary ribs in animal models, and (iv) 3D biofabrication of bone development and regeneration tissue models. In addition, increased collaboration with the medical community was suggested to improve the relevance of test results to humans and identify more clinically relevant endpoints. Finally, the participants agreed that this conference facilitated better understanding innovative approaches that can be useful for the identification of developmental health risks due to exposure to chemical substances.

Keywords: DevTox-project, Grey zone anomalies, neurodevelopmental toxicology testing

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Inamori G*1, Kamoto U*1, Nakamura F*1, Isoda Y*1, Uozumi A*2, Matsuda R*1, Shimamura M*1, Okubo Y, Ito S*2, Ota H*1: Neonatal wearable device for colorimetry-based real-time detection of jaundice with simultaneous sensing of vitals.

Science Advances 2021;7(10):eabe3793. doi: 10.1126/sciadv.abe3793

Neonatal jaundice occurs in >80% of newborns in the first week of life owing to physiological hyperbilirubinemia. Severe hyperbilirubinemia could cause brain damage owing to its neurotoxicity, a state commonly known as kernicterus. Therefore, periodic bilirubin monitoring is essential to identify infants at-risk and to initiate treatment including phototherapy. However, devices for continuous measurements of bilirubin have not been developed yet. Here, we established a wearable transcutaneous bilirubinometer that also has oxygen saturation (SpO2) and heart rate (HR) sensing functionalities. Clinical experiments with neonates demonstrated the possibility of simultaneous detection of bilirubin, SpO2, and HR. Moreover, our device could consistently measure bilirubin during phototherapy. These results demonstrate the potential for development of a combined treatment approach with an automatic link via the wearable bilirubinometer and phototherapy device for optimization of the treatment of neonatal jaundice.

Keywords: neonatal wearable device, colorimetry-based real-time detection, simultaneous sensing of vitals

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Yokota S, Sekine N*1, Wakayama T*2, Oshio S*1: Impact of chronic vitamin A excess on sperm morphogenesis in mice.

Andrology. 2021; doi:10.1111/andr.13013

Background: The increasing availability of fortified foods and supplements have caused an overconsumption of vitamin A (VA), above the recommended level. To date, the effects of chronic VA excess (VAE) on spermatogenesis remains unclear.

Objective: This study aims to investigate the long-term excessive intake of VA effects on spermatogenesis in mice.

Materials and methods: Dams were initially fed a control diet (4 IU/g) or a VAE diet (250 IU/g), 4 weeks prior to matting and during pregnancy. Dams and their male pups continued this diet regimen until the offspring reached 12 weeks of age. At 12 weeks of age, epididymis caudal spermatozoa and testes were collected. For histological analysis, sections were stained with periodic acid-Schiff-hematoxylin, and quantitative PCR was used to detect changes in gene expression in the testes of the VAE mice. Sperm motility and morphology were evaluated to detect the endpoint of VAE toxicity.

Results: Body weights were not significantly different between the control and VAE groups. Testicular cross-sections from the control and VAE mice contained a normal array of germ cells, and the daily sperm production was similar between the two groups. However, the percentage of seminiferous tubules in stages VII and VIII was significantly lower in the VAE mice than in the control. In addition, significant changes in the expression of genes involved in retinoid metabolism, spermatogenesis, and spermiogenesis were detected in the testes of the VAE mice. Consistently, sperm motility and head morphology were significantly impaired in the VAE mice.

Discussion and Conclusion: Our findings suggest that long-term dietary intake of VAE were able to influence both pre- and post-meiotic spermatogenesis. As a result of testicular toxicity, we demonstrated, to the best of our knowledge, for the first time that long-term VAE caused sperm-head abnormalities.

Keywords: reproductive toxicology, spermatogenesis, vitamin A

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Figarol A*1, Naka Y*1, Shigemoto-Mogami Y, Furihata T*2, Sato K, Matsusaki M*1: In Vitro self-organized three-dimensional model of the blood-brain barrier microvasculature.
Biomed Mater. 2020. doi: 10.1088/1748-605X/aba5f1.

The blood-brain barrier (BBB) protects the human brain from external aggressions. Despite it great importance, very few in vitro models of the BBB reproducing its complex organization are yet available. Here we fabricated such a three-dimensional (3D) self-organized in vitro model of BBB microvasculature by means of collagen microfibers (CMF) and fibrin gel combination. The interconnected fibres supported human brain microvascular endothelial cell migration and the formation of a capillary-like network with lumen diameter close to in vivo values. Fibrin, a protein involved in blood vessel repair, favored further the 3D conformation of brain microvascular endothelial cells, astrocytes and pericytes, ensured gel cohesion and avoided shrinkage. The maturation of the BBB microvasculatures network was stimulated by both the CMF and the fibrin in the hydrogel. Expression of essential tight junction proteins, carriers and transporters were validated in regards to bidimensional simple coculture. The volume of gel drops was easily tunable to fit in 96 well-plates. D-Mannitol cytotoxicity and impacts on microvascular network were evaluated, as an example of the pertinence of this 3D BBB capillary model for screening applications.
Keywords: blood-brain barrier, self-organized 3D microvasculature; tissue engineering

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Irie T, Yamazaki D, Kikura-Hanajiri R: F-phenibut (beta-(4-Fluorophenyl)-GABA), a potent GABAB receptor agonist, activates an outward-rectifying K(+) current and suppresses the generation of action potentials in mouse cerebellar Purkinje cells.
Eur J Pharmacol 2020;884:173437-45. doi: 10.1016/j.ejphar.2020.173437

The GABA analog phenibut (beta-Phenyl-GABA) is a GABAB receptor agonist that has been licensed for various uses in Russia. Phenibut is also available as a dietary supplement from online vendors worldwide, and previous studies have indicated that phenibut overdose results in intoxication, withdrawal symptoms, and addiction. F-phenibut (beta-(4-Fluorophenyl)-GABA), a derivative of phenibut, has not been approved for clinical use. However, it is also available as a nootropic supplement from online suppliers. F-phenibut binds to GABAB with a higher affinity than phenibut; therefore, F-phenibut may lead to more serious intoxication than phenibut. However, the mechanisms by which F-phenibut acts on GABAB receptors and influences neuronal function remain unknown. In the present study, we compared the potency of F-phenibut, phenibut, and the GABAB agonist (+/−)-baclofen (baclofen) using in vitro patch-clamp recordings obtained from mouse cerebellar Purkinje cells slice preparations Our findings indicate that F-phenibut acted as a potent GABAB agonist. EC50 of outward current density evoked by the three GABAB agonists decreased in the following order: phenibut (1362 muM) > F-phenibut (23.3 muM) > baclofen (6.0 muM). The outward current induced by GABAB agonists was an outward-rectifying K(+) current, in contrast to the previous finding that GABAB agonists activates an inward-rectifying K(+) current. The K(+) current recorded in the present study was insensitive to extracellular Ba(2+), intra- or extracellular Cs(+), and intra- or extracellular tetraethylammonium-Cl. Moreover, F-phenibut suppressed action potential generation in Purkinje cells. Thus, abuse of F-phenibut may lead to severe damage by inhibiting the excitability of GABAB-expressing neurons.
Keywords: F-phenibut, phenibut, GABAB

Izumi-Nakaseko H*, Chiba K*, Hagiwara-Nagasawa M*, Satsuka A, Goto A*, Nunoi Y*, Kambayashi R*, Matsumoto A*, Takei Y*, Kanda Y, Naito AT*, Sugiyama A*: Optimizing the Direction and Order of the Motion Unveiled the Ability of Conventional Monolayers of Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes to Show Frequency-Dependent Enhancement of Contraction and Relaxation Motion.
Frontiers in Cell and Developmental Biology.

2020;8:542562. doi: 10.3389/fcell.2020.542562

Contractility of the human heart increases as its beating rate is elevated, so-called positive force-frequency relationship; however, human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) have been reported to exert a negative force-frequency relationship. We tested the hypothesis that the regulation of motion directions by electrical pacing and/or oxygen supply may improve the electro-mechanical properties of hiPSC-CMs monolayers. To better evaluate the spatial and temporal relationship between electrical excitation and contractile motion, we simultaneously observed the field potential and motion vector of hiPSC-CMs sheets. Under spontaneous contraction, although an electrical excitation originating from a region propagated unidirectionally over the cell sheet, contraction wave started from multiple sites, and relaxation wave was initiated from a geometric center of hiPSC-CMs sheet. During electrical pacing, contraction and relaxation waves were propagated from the stimulated site. Interestingly, the maximum contraction speed was more increased when the hiPSC-CMs sheet was stimulated at an area relaxation initiated under spontaneous condition. Furthermore, motion vector analysis demonstrated that "positive contraction velocity-frequency relationship" in contraction and "frequency-dependent enhancement of relaxation" were produced in the cell sheet by optimizing the direction and order of the contractile motion with pacing at the relaxation-initiating area. A close analysis of motion vectors along with field potential recording demonstrated that relaxation process consists of fast and slow phases, and suggest that intracellular Ca^{2+} dynamics may be closely related to functions of Ca^{2+}-ATPase pump and Na^{+}-Ca^{2+} exchangers. Namely, the slow relaxation phase occurred after the second peak of field potential, suggesting that the slow phase may be associated with extrusion of Ca^{2+} by Na^{+}-Ca^{2+} exchangers during repolarization. Increase of oxygen concentration from 20 to 95% as well as β-adrenergic stimulation with isoproterenol accelerated the fast relaxation, suggesting that it could depend on Ca^{2+} uptake via Ca^{2+}-ATPase during the depolarization phase. The ratio of maximum contraction speed to field potential duration was increased by the β-adrenergic stimulation, indicating the elevated contraction efficiency per Ca^{2+}-influx. Thus, these findings revealed potential ability of conventional monolayers of hiPSC-CMs, which will help apply them to translational study filling the gap between physiological as well as pharmacological studies and clinical practice.

Keywords: contraction velocity-frequency relationship, field potential, frequency-dependent enhancement of relaxation

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Goto A[*1], Sakamoto K[*2], Kambayashi R[*1], Nunoi Y[*1], Izumi-Nakaseko H[*1], Kawai S[*1], Takei Y[*1], Matsumoto A[*1], Kanda Y, Sugiyama A[*1]: Torsadogenic action of cisapride, dl-sotalol, bepridil and verapamil analyzed by the chronic atrioventricular block cynomolgus monkeys: Comparison with that reported in the CiPA in silico mechanistic model.

Toxicological Sciences. 2021;181:125-133. doi: 10.1093/toxsci/kfab015

In order to bridge the gap of information between the in silico model and human subjects, we evaluated torsadogenic risk of cisapride, dl-sotalol, bepridil and verapamil selected from 12 training compounds in the comprehensive in vitro proarrhythmia assay using the chronic atrioventricular block monkeys. Cisapride (0, 1, and 5 mg/kg, n = 5 for each dose), dl-sotalol (0, 1, 3, and 10 mg/kg, n = 5 for each dose), bepridil (0, 10, and 100 mg/kg, n = 4 for each dose), verapamil (0, 1.5, 15, and 75 mg/kg, n = 4 for each dose) were orally administered to the monkeys in conscious state. Five mg/kg of cisapride, 1, 3, and 10 mg/kg of dl-sotalol and 100 mg/kg of bepridil prolonged ΔΔQTcF, which was not observed by verapamil. Torsade de pointes was induced by 5 mg/kg of cisapride in 2 out of 5 animals, by 10 mg/kg of dl-sotalol in 5 out of 5 and by 100 mg/kg of bepridil in 2 out of 4, which was not induced by verapamil. These torsadogenic doses were normalized by their maximum clinical daily ones to estimate torsadogenic risk. The order of risk was dl-sotalol >bepridil ≥cisapride >verapamil in our study. Since the order was bepridil ≥dl-sotalol >cisapride >verapamil in comprehensive in vitro proarrhythmia assay (CiPA) in silico mechanistic model validation, sympathetic regulation on the heart may play a pivotal role in the onset of torsade de pointes in vivo.

Keywords: in silico model, CiPA, atrioventricular block.

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Fukushima H*1, Yoshioka M*1, Kawatou M*1,2, López-Dávila V*1, Takeda M*1, Kanda Y, Sekino Y*3, Yoshida Y*1, Yamashita JK*1: Specific induction and long-term maintenance of high purity ventricular cardiomyocytes from human induced pluripotent stem cells.
PLoS One. 2020;15:e0241287. doi: 10.1371/journal.pone.0241287

Currently, cardiomyocyte (CM) differentiation methods require a purification step after CM induction to ensure the high purity of the cell population. Here we show an improved human CM differentiation protocol with which high-purity ventricular-type CMs can be obtained and maintained without any CM purification process. We induced and collected a mesodermal cell population (PDGFR α-positive cells) that can respond to CM differentiation cues, and then stimulated CM differentiation by means of Wnt inhibition. This method reproducibly generated CMs with purities above 95% in several human pluripotent stem cell lines. Furthermore, these CM populations were maintained in culture at such high purity without any further CM purification step for over 200 days. The majority of these CMs (>95%) exhibited a ventricular-like phenotype with a tendency to structural and electrophysiological maturation, including T-tubule-like structure formation and the ability to respond to QT prolongation drugs. This is a simple and valuable method to stably generate CM populations suitable for cardiac toxicology testing, disease modeling and regenerative medicine.
Keywords: cardiomyocyte differentiation, human pluripotent stem cell, ventricular-like phenotype

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Kamata S*1, Hashiyama R*1, Hana-Ika H*1, Ohkubo I*1, Saito R*1, Honda A*1, Anan Y*1, Akahoshi N*1, Noguchi K*2, Kanda Y, Ishii I*1: Cytotoxicity comparison of 35 developmental neurotoxicants in human induced pluripotent stem cells (iPSC), iPSC-derived neural progenitor cells, and transformed cell lines.
Toxicology in Vitro. 2020;69:104999. doi: 10.1016/j.tiv.2020.104999

The Organization for Economic Co-operation and Development (OECD) test guideline 426 for developmental neurotoxicity (DNT) of industrial/environmental chemicals depends primarily on animal experimentation. This requirement raises various critical issues, such as high cost, long duration, the sacrifice of large numbers of animals, and interspecies differences. This study demonstrates an alternative protocol that is simple, quick, less expensive, and standardized to evaluate DNT of many chemicals using human induced pluripotent stem cells (iPSC) and their differentiation to neural progenitor cells (NPC). Initially, concentration-dependent cytotoxicity of 35 DNT chemicals, including industrial materials, insecticides, and clinical drugs, were compared among iPSC, NPC, and two transformed cells, Cos-7 and HepG2, using tetrazolium dye (MTS)-reducing colorimetric and ATP luciferase assays, and IC50 values were calculated. Next, inhibitory effects of the 14 representative chemicals (mainly insecticides) on iPSC differentiation to NPC were evaluated by measuring altered expression of neural differentiation and undifferentiation marker genes. Results show that both iPSC and NPC were much more sensitive to most DNT chemicals than the transformed cells, and 14 chemicals induced differential patterns of marker gene expression, highlighting the validity and utility of the protocol for evaluation and classification of DNT chemicals and preclinical DNT tests for safety assessment.
Keywords: cell viability assay, cytotoxicity, developmental neurotoxicity

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Tsukada K*1, Shinki S*1, Kaneko A*1, Murakami K*2, Irie K*2, Murai M*2, Miyoshi H*2, Dan S*3, Kawaji, K*4, Hayashi H*4, Kodama NE*4, Hori A*5, Salim E*5, Kuraishi T*5, Hirata N, Kanda Y, Asai T*1,4: Synthetic biology based construction of biological activity-related library of fungal decalin-containing

diterpenoid pyrenes.

Nature Communications. 2020;11:1830. doi: 10.1038/s41467-020-15664-4.

A synthetic biology method based on heterologous biosynthesis coupled with genome mining is a promising approach for increasing the opportunities to rationally access natural product with novel structures and biological activities through total biosynthesis and combinatorial biosynthesis. Here, we demonstrate the advantage of the synthetic biology method to explore biological activity-related chemical space through the comprehensive heterologous biosynthesis of fungal decalin-containing diterpenoid pyrones (DDPs). Genome mining reveals putative DDP biosynthetic gene clusters distributed in five fungal genera. In addition, we design extended DDP pathways by combinatorial biosynthesis. In total, ten DDP pathways, including five native pathways, four extended pathways and one shunt pathway, are heterologously reconstituted in a genetically tractable heterologous host, Aspergillus oryzae, resulting in the production of 22 DDPs, including 15 new analogues. We also demonstrate the advantage of expanding the diversity of DDPs to probe various bioactive molecules through a wide range of biological evaluations.

Keywords: genetic engineering, metabolic engineering, combinatorial library

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Sasaki H*1, Masuno H*2, Kawasaki H*1, Yoshihara A*1, Numoto N*2, Ito N*2, Ishida H*3, Yamamoto K*3, Hirata N, Kanda Y, Kawachi E*2, Kagechika H*2, Tanatani A*1: Lithocholic Acid Derivatives as Potent Vitamin D Receptor Agonists.

Journal of Medicinal Chemistry. 2021;64:516-526. doi: 10.1021/acs.jmedchem.0c01420

Lithocholic acid (2) was identified as a second endogenous ligand of vitamin D receptor (VDR), though its activity is very weak. In this study, we designed novel lithocholic acid derivatives based on the crystal structure of VDR-ligand-binding domain (LBD) bound to 2. Among the synthesized compounds, 6 bearing a 2-hydroxy-2-methylprop-1-yl group instead of the 3-hydroxy group at the 3α-position of 2 showed dramatically increased activity in HL-60 cell differentiation assay, being at least 10 000 times more potent than lithocholic acid (2) and 3 times more potent than 1α,25-dihydroxyvitamin D3 (1). Although the binding affinities of 6 and its epimer 7 were less than that of 1, their transactivation activities were greater than that of 1. X-ray structure analyses of VDR LBD bound to 6 or 7 showed that the binding positions of these compounds in the ligand-binding pocket are similar to that of 1.

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Yamada T, Toyoda T, Matsushita K, Cho YM, Akagi J, Morikawa T, Mizuta Y, Ogawa K: Expression of stem cell markers as useful complementary factors in the early detection of urinary bladder carcinogens by immunohistochemistry for γ-H2AX.

Arch Toxicol. 2021;95:715-26. doi: 10.1007/s00204-020-02950-9.

We previously demonstrated that immunohistochemistry for γ-H2AX, a biomarker of DNA damage, is useful for early detection of urinary bladder carcinogens in rats. In a 28-day repeated-dose study, γ-H2AX was shown to have high sensitivity for detection of bladder carcinogens. However, no reports have evaluated whether a combination of multiple biomarkers may further improve sensitivity. Accordingly, in this study, we aimed to evaluate the applicability of bladder tissue and cancer stem cell markers, including cytokeratin 14 (KRT14), aldehyde dehydrogenase 1A1 (ALDH1A1), and cluster of differentiation 44 (CD44), as complementary markers for early detection of bladder carcinogens. Bladder samples obtained from male F344 rats orally treated with 14 bladder carcinogens and 5 nonbladder carcinogens for 28 days were used for immunohistochemical analysis of stem cell markers. In the bladder carcinogen-treated rats, increases in KRT14, ALDH1A1, and CD44 expression were observed in 9, 10, and 10 out of 14 groups, respectively, whereas the 5 nonbladder carcinogens did not cause upregulation of these markers. Although most

epithelial cells with KRT14 or ALDH1A1 expression were also positive for CD44, KRT14 and ALDH1A1 expression were mutually exclusive. Twelve bladder carcinogens showed increases in at least one of the three markers, indicating that the combined evaluation showed higher sensitivity than the use of individual markers alone. Importantly, 2 of 3 bladder carcinogens that did not induce γ-H2AX immunostaining showed stem cell marker expression. Our results demonstrated that these stem cell markers may be useful as complementary markers for γ-H2AX in evaluation of bladder carcinogens.

Keywords: γ-H2AX, stem cell, urinary bladder

Kobayashi T[*1], Toyoda T, Tajima Y[*1], Kishimoto S[*1], Tsunematsu Y[*1], Sato M[*1], Matsushita K, Yamada T, Shimamura Y[*1], Masuda S[*1], Ochiai M[*1], Ogawa K, Watanabe K[*1], Takamura-Enya T[*2], Totsuka Y[*3], Wakabayashi K[*1], Miyoshi N[*1]: *o*-Anisidine dimer, 2-methoxy-*N*[4]-(2-methoxyphenyl) benzene-1,4-diamine, in rat urine associated with urinary bladder carcinogenesis.

Chem Res Toxicol. 2021;34:912-9. doi: 10.1021/acs.chemrestox.0c00536.

Monocyclic aromatic amines, *o*-toluidine (*o*-Tol) and its structural analog *o*-anisidine (*o*-Ans) are IARC Group 1 and Group 2A urinary bladder carcinogens, respectively, and are involved in metabolic activation and DNA damage. Our recent study revealed that 2-methyl-*N*[4]-(2-methylphenyl) benzene-1,4-diamine (MMBD), a psemidine-type homodimer of *o*-Tol, was detected and identified in an in vitro reaction of *o*-Tol with S9 mix and *in vivo* urinary samples of *o*-Tol-exposed rats. Potent mutagenic, genotoxic, and cytotoxic activities were reported with MMBD, suggesting its involvement in urinary bladder carcinogenesis. However, it remains unknown whether *o*-Ans is converted to active metabolites to induce DNA damage in a similar manner as *o*-Tol. In this study, we report that a novel *o*-Ans metabolite, 2-methoxy-*N*[4]-(2-methoxyphenyl) benzene-1,4-diamine (MxMxBD), a dimer by head-to-tail binding (psemidine form), was for the first time identified in *o*-Ans-exposed rat urine. MxMxBD induced a stronger mutagenicity in *N*-acetyltransferase overexpressed *Salmonella typhimurium* strains, and potent genotoxicity and cytotoxicity in human bladder carcinoma T24 cells compared with *o*-Ans. These results suggest that MxMxBD may to some extent contribute toward urinary bladder carcinogenesis. In addition to homodimerization, such as MxMxBD, heterodimerizations were observed when *o*-Ans was coincubated with *o*-Tol or aniline (Ani) in *in vitro* reactions with S9 mix. This study highlights the important consideration of homo- and heterodimerizations of monocyclic aromatic amines, including *o*-Ans, *o*-Tol, and Ani, in the evaluation of the combined exposure risk of bladder carcinogenesis.

Keywords: aromatic amine, carcinogenesis, urinary bladder

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Tajima Y[*1], Toyoda T, Hirayama Y[*1], Matsushita K, Yamada T, Ogawa K, Watanabe K[*1], Takamura-Enya T[*1], Totsuka Y[*2], Wakabayashi K[*1], Miyoshi N[*1]: A novel *o*-toluidine metabolite in rat urine associated with urinary bladder carcinogenesis.

Chem Res Toxicol. 2020;33:1907-14. doi: 10.1021/acs.chemrestox.0c00098.

ortho-Toluidine (*o*-Tol), a monocyclic aromatic amine, causes bladder cancers in human and experimental animals, and is therefore classified as a Group 1 carcinogen (IARC), in which the carcinogenicity of *o*-Tol is involved in metabolic activation, DNA damage and DNA adduct formation. In the DNA adduct formation mechanism, *o*-Tol is metabolized by *N*-hydroxylation, *N*-acetoxylation, and then deacetoxylation to produce an electrophilic nitrenium ion, which is able to bind to a DNA base, such as dG-C8. Therefore dG-C8-*o*-Tol is thought to be a plausible DNA adduct of *o*-Tol exposure. However direct detection of dG-C8-*o*-Tol in biological samples has not been reported yet. Here we show that a novel *o*-Tol metabolite, 2-methyl-*N*[1]-(2-methylphenyl) benzene-1,4-diamine (MMBD), a dimer by head-to-tail binding, was identified for the first time in *o*-Tol-exposed rat urine. MMBD was also detected in a reaction of *o*-Tol and S9 mix, indicating the formation was catalyzed by an enzymatic reaction. Moreover, MMBD showed a potent stronger mutagenicity in *N*-acetyltransferase overexpressed Salmonella typhimurium strains, and

cytotoxicity in human bladder carcinoma T24 cells and human spleen lymphoblastoid TK6 cells compared with *o*-Tol. Furthermore, a DNA adduct (*m/z* 478.1) corresponding to dG-MMBD was detected in the reaction of calf thymus DNA with rat urine containing MMBD, and also in hepatic DNA of rats treated with *o*-Tol. These results therefore suggested that *o*-Tol-induced bladder carcinogenesis could be at least partly attributed to MMBD formation. The possible dimerization of monocyclic aromatic amines should be considered in the evaluation of the risk of bladder carcinogenesis following exposure.

Keywords: aromatic amine, carcinogenesis, urinary bladder

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Nakamura K, Ishii Y, Takasu S, Ogawa K: A 90-day subchronic toxicity study of 5-methyl-2-phenyl-2-hexenal in F344 rats.
Food Chem Toxicol. 2021;150:112041. doi: 10.1016/j.fct.2021.112041.

5-Methyl-2-phenyl-2-hexenal (MPH) has been used as a flavoring agent. In the present study, we performed a subchronic toxicity study in male and female F344 rats with oral administration of MPH by gavage at 0, 8, 24 and 70 mg/kg body weight (BW)/day for 90 days. No mortality or clinical signs were observed during the experimental period. Body weight and food consumption for all treated groups of both sexes were essentially the same as for the respective control groups. Hematologic examination demonstrated significant decreases in monocyte counts for females given 24 and 70 mg/kg BW/day. However, these changes were not substantial and no related histopathological changes were observed, suggesting that these changes were not toxicologically significant. Among organ weights, the absolute and/or relative weights of testes and liver were significantly increased in the 70 mg/kg BW/day groups of males and females, respectively, but no related histopathological changes were observed, suggesting that these changes did not reflect adverse effects. In addition, no treatment-related histopathological changes were observed for any of the tissues examined. Based on the overall data, the no-observed-adverse-effect level (NOAEL) for MPH was determined to be 70 mg/kg BW/day, the highest dose tested, in both male and female rats.

Keywords: 5-methyl-2-phenyl-2-hexenal, flavoring agent, subchronic toxicity

Matsushita K, Toyoda T, Yamada T, Morikawa T, Ogawa K: Comprehensive expression analysis of mRNA and microRNA for investigation of compensatory mechanisms in the rat kidney after unilateral nephrectomy.
J Appl Toxicol. 2020;40:1373-83. doi: 10.1002/jat.3990.

Compensation is a physiological response that occurs during chemical exposure to maintain homeostasis. Because compensatory responses are not usually considered adverse effects, it is important to understand compensatory mechanisms for chemical risk assessment. Although the kidney is a major target organ for toxicity, there is controversy over whether hyperplasia or hypertrophy contributes to the compensatory mechanism, and there is limited information to apply for chemical risk assessment. In the current study, compensatory mechanisms of the kidney were investigated in a unilateral nephrectomy (UNx) model using adult male and female F344 rats. In residual kidneys of male and female rats after UNx, 5-bromo-2'-deoxyuridine-labeling indices and mRNA expression of cell cycle-related genes were increased, although there were no fluctuations in mRNA expression of transforming growth factor-β1, which contributes to hypertrophy in renal tubules. Pathway analysis using mRNA expression data from a cDNA microarray revealed that canonical pathways related to cell proliferation were mainly activated and that forkhead box M1 (FOXM1) was an upstream regulator of compensatory cell proliferation in residual kidneys of male and female rats. cDNA microarray for microRNAs (miRNAs) demonstrated that 9 miRNAs were downregulated in residual kidneys, and mRNA/miRNA integrated analysis indicated that miRNAs were associated with the expression of factors downstream of FOXM1. Overall, these results suggested that FOXM1-mediated hyperplasia rather than hypertrophy contributed to compensatory mechanisms in the kidney and that miRNAs regulated downstream FOXM1 signaling. These results will be beneficial for evaluating nephrotoxicity in chemical risk assessment and for developing new biomarkers to

predict nephrotoxicity.

Keywords: renal compensation, compensatory hyperplasia, unilateral nephrectomy

Yamada T, Toyoda T, Matsushita K, Morikawa T, Ogawa K: Dose dependency of γ-H2AX formation in the rat urinary bladder treated with genotoxic and nongenotoxic bladder carcinogens.

J Appl Toxicol. 2020;40:1219-27. doi: 10.1002/jat.3978.

We previously reported that immunostaining for γ-H2AX, a biomarker of DNA damage, in the rat urinary bladder is useful for early detection of bladder carcinogens in 28-day toxicity studies. Here, we aimed to examine the dose dependency of γ-H2AX formation in the urinary bladder of rats. Male F344 rats (aged 6 weeks) were orally administered *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN; 0%, 0.0001%, 0.001%, 0.01%, 0.02%, or 0.05% in drinking water), a genotoxic bladder carcinogen, and melamine (0%, 0.3%, 1.0%, or 3.0% in the diet), a nongenotoxic bladder carcinogen, for 2 days or 4 weeks. Immunohistochemical analysis showed that γ-H2AX- and Ki67-positive epithelial cells in the bladder urothelium were significantly increased, with a clear dose dependency, in both BBN- and melamine-treated groups. Additionally, γ-H2AX formation was detected from the lower-dose group, without increased Ki67 expression or histopathologic findings. The ratios of γ-H2AX-positive cells at week 4 in both BBN- and melamine-treated groups were higher than those on day 2, indicating the time-dependent increase in γ-H2AX formation. Immunofluorescence double-staining revealed that γ-H2AX single-positive cells without Ki67 expression were often found in the urothelium of BBN-treated rats, whereas most γ-H2AX-positive cells were Ki67-positive in the melamine group. Our results demonstrated that γ-H2AX formation in the urinary bladder increased in a clear dose-dependent manner and that γ-H2AX immunostaining has the potential to detect bladder carcinogens after a 2-day administration. Furthermore, the association of genotoxic mechanisms in bladder carcinogenesis could be determined by analyzing the colocalization of γ-H2AX and Ki67 in the urothelium.

Keywords: γ-H2AX, carcinogenicity, urinary bladder

Yamada T, Toyoda T, Ide T, Matsushita K, Morikawa T, Ogawa K: Neuromuscular and vascular hamartoma of the small intestine in an F344 rat.

J Toxicol Pathol. 2021;34:113-7. doi: 10.1293/tox.2020-0059.

An intestinal mass was found in the border area of the jejunum and ileum of a 110-week-old male F344 rat. Histopathologically, the mass protruded into the lumen and was covered with intestinal epithelium, exhibiting a normal architecture. The lesion was located in the submucosa and consisted of loose connective tissue, smooth muscle, scattered ganglion cells, and blood vessels of various sizes. Although these components showed an irregular and disordered structure, no cellular atypia, increased proliferation activity, or invasive growth to adjacent tissues were detected. Immunohistochemical analyses revealed that smooth muscle, ganglion, and endothelial cells were positive for α-smooth muscle actin and vimentin, S-100, and CD34 and von Willebrand factor, respectively, indicating maturation of these cells. Thus, the mass was diagnosed as a neuromuscular and vascular hamartoma of the small intestine. To the best of our knowledge, this is the first report of this type of lesion in rodents.

Keywords: hamartoma, small intestine, case report

Matsushita K, Ishii Y, Kijima A, Takasu S, Kuroda K, Takagi H*, Nohmi T, Ogawa K, Umemura T: Background data of 2-year-old male and female F344 *gpt* delta rats.

J Toxicol Pathol. 2021;34:23-31. doi: 10.1293/tox.2020-0060.

Although *gpt* delta rats, as reporter gene-transgenic rats, were originally developed for *in vivo* mutation assays, they have also been used to evaluate chemical carcinogenesis and comprehensive toxicity. Therefore, it is necessary to accumulate background data on carcinogenicity and general toxicity in *gpt* delta rats. Here, we investigated the background data of 110-week-old male and female F344 *gpt* delta rats and wild-type rats. There was no effect of reporter gene transfection on animal survival rates and body weights during the experiment. The relative weight of male *gpt* delta rat adrenals was significantly higher than that of wild-type rats, possibly due to the higher incidence of pheochromocytoma. There were no intergenotype differences in the incidence of nonneoplastic lesions in

both sexes, including chronic progressive nephropathy and focus of cellular alteration in the liver, which had a higher incidence in both genotypes. Additionally, the significantly higher incidence of adrenal pheochromocytoma in male *gpt* delta rats than that in wild-type rats was likely incidental because of the lack of differences in the incidences of preneoplastic (male and female) and neoplastic (female) adrenal lesions in both genotypes. Other neoplastic lesions in both sexes showed no intergenotype differences in incidence rates, although large granular lymphocytic leukemia in the spleen and Leydig cell tumors in the testes of males showed higher incidence rates. Overall, there were no effects of reporter gene transfection on the spectrum of spontaneous lesions in F344 *gpt* delta rats, thus supporting their applicability in evaluating chemical toxicity and carcinogenicity.

Keywords: *gpt* delta rat, F344 rat, background data

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Suzuki S[*1], Gi M[*1], Toyoda T, Kato H[*2], Naiki-Ito A[*2], Kakehashi A[*1], Ogawa K, Takahashi S[*2], Wanibuchi H[*1]: Role of γ-H2AX as a biomarker for detection of bladder carcinogens in F344 rats.

J Toxicol Pathol. 2020;33:279-85. doi: 10.1293/tox.2020-0038.

Phosphorylation of histone H2AX at serine 139 (γ-H2AX) is known to be induced by direct DNA damage or cellular metabolic imbalances and malfunctions. Previous studies have reported that γ-H2AX is a useful biomarker for early detection of genotoxic bladder carcinogens in rats. The purpose of the present study was to determine the role of γ-H2AX as a biomarker for detection of non-genotoxic bladder carcinogens in rats. Six-week-old male F344 rats were treated with 15 different chemicals for 4 weeks. Immunohistochemical analyses revealed that all three genotoxic bladder carcinogens and six out of seven non-genotoxic bladder carcinogens significantly increased γ-H2AX formation in the bladder urothelium of rats. In addition, four out of five rat bladder noncarcinogens did not increase γ-H2AX formation in the bladder urothelium regardless of genotoxicity. These results suggest that γ-H2AX is a useful biomarker for detection of both genotoxic and non-genotoxic bladder carcinogens in rats.

Keywords: γ-H 2 AX, carcinogenicity, urinary bladder

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Ide T, Mizuta Y, Akagi J, Masumoto N, Sugimoto N, Sato K, Ogawa K, Cho YM: A 90-day repeated oral dose toxicity study of four stereoisomers of 2,4-dimethyl-4-phenyltetrahydrofuran, a synthetic flavoring substance, in F344 rats.

Regul Toxicol Pharmacol. 2020;114:104664. doi: 10.1016/j.yrtph.2020.104664.

2,4-Dimethyl-4-phenyltetrahydrofuran (CAS no. 82461-14-1) is a food additive used as a synthetic flavoring substance. To investigate the toxicological properties and determine the no-observed-adverse-effect level (NOAEL), a 90-day repeated oral dose toxicity study of 2,4-dimethyl-4-phenyltetrahydrofuran containing four stereoisomers was conducted in F344 rats at doses of 0, 6, 24, and 96 mg/kg body weight (BW)/day. No mortality or abnormal clinical signs related to treatment in any group was observed. At a dose of 96 mg/kg BW, serum total protein and total cholesterol and increased absolute and relative liver and kidney weights were observed in both sexes. Increased serum albumin in males and decreased Na and Cl in females were also observed. On histopathological assessment, at a dose of 96 mg/kg BW, diffuse hepatocellular hypertrophy in the liver in both sexes and tubular regeneration with scattered proximal tubular degeneration and/or necrosis throughout the cortex in the kidney were detected in males. Based on these findings, the NOAEL for 2,4-dimethyl-4-phenyltetrahydrofuran used in the current study was found to be 24 mg/kg BW/day for both sexes.

Keywords: food additive, subchronic toxicity, rat

Sakai W[*1], Yuasa-Sunagawa M[*1], Kusakabe M[*1], Kishimoto A[*1], Matsui T[*1], Kaneko Y[*1], Akagi J, Huyghe N[*2], Ikura M[*3], Ikura T[*3], Hanaoka F[*4], Yokoi M[*1], Sugasawa K[*1]: Functional impacts of the ubiquitin–proteasome system on DNA damage recognition in global genome nucleotide excision repair.

Sci Rep. 2020;10:19704. doi: s41598-020-76898-2.

The ubiquitin–proteasome system (UPS) plays

crucial roles in regulation of various biological processes, including DNA repair. In mammalian global genome nucleotide excision repair (GG-NER), activation of the DDB2-associated ubiquitin ligase upon UV-induced DNA damage is necessary for efficient recognition of lesions. To date, however, the precise roles of UPS in GG-NER remain incompletely understood. Here, we show that the proteasome subunit PSMD14 and the UPS shuttle factor RAD23B can be recruited to sites with UV-induced photolesions even in the absence of XPC, suggesting that proteolysis occurs at DNA damage sites. Unexpectedly, sustained inhibition of proteasome activity results in aggregation of PSMD14 (presumably with other proteasome components) at the periphery of nucleoli, by which DDB2 is immobilized and sequestered from its lesion recognition functions. Although depletion of PSMD14 alleviates such DDB2 immobilization induced by proteasome inhibitors, recruitment of DDB2 to DNA damage sites is then severely compromised in the absence of PSMD14. Because all of these proteasome dysfunctions selectively impair removal of cyclobutane pyrimidine dimers, but not (6–4) photoproducts, our results indicate that the functional integrity of the proteasome is essential for the DDB2-mediated lesion recognition sub-pathway, but not for GG-NER initiated through direct lesion recognition by XPC.

Keywords: DNA repair, ubiquitin, proteasome

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Takasu S, Ishii Y, Kijima A, Ogawa K, Nakane S, Umemura T: Furan induced characteristic glutathione *S*-transferase placental form-positive foci in terms of cell kinetics and gene expression.
Toxicol Pathol. 2020;48:756-65. doi: 10.1177/0192623320948782.

Glutathione *S*-transferase placental form-positive (GST-P$^+$) foci are markers of preneoplastic lesions in rat hepatocarcinogenesis. Our previous studies using reporter gene transgenic rats showed that furan, a hepatocarcinogen in rodents, rapidly induces the formation of GST-P$^+$ foci after short exposure without reporter gene mutation. We hypothesized that GST-P$^+$ foci induced by furan may have biological characteristics different from those induced by diethylnitrosamine (DEN), a genotoxic hepatocarcinogen. Accordingly, we compared the cell kinetics of GST-P$^+$ foci after cessation of DEN treatment and performed comprehensive gene expression in DEN- or furan-induced GST-P$^+$ foci. The number and area of DEN-induced GST-P$^+$ foci were increased after cessation of treatment, whereas furan decreased these parameters. Size distribution analysis showed that large furan-induced GST-P$^+$ foci disappeared after cessation of treatment. Hierarchical cluster analysis showed that all samples from GST-P$^+$ foci induced by furan were separated from those induced by DEN. SOX9 expression was upregulated in furan-induced GST-P$^+$ foci and was detected by immunohistochemistry in large furan-induced GST-P$^+$ foci. Our results indicated that large furan-induced GST-P$^+$ foci were quite different from DEN-induced GST-P$^+$ foci at the molecular and cellular levels. And one of the properties of disappearing large GST-P$^+$ foci were characterized by inclusion of hepatocytes expressing SOX9.

Keywords: SOX9, GST-P, hepatocarcinogen

Nakamura K, Ishii Y, Takasu S, Nohmi T, Shibutani M*, Ogawa K: Lack of *in vivo* mutagenicity of acetamide in a 13-week comprehensive toxicity study using F344 *gpt* delta rats.
Toxicol Sci. 2020;177:431-40. doi: 10.1093/toxsci/kfaa126.

Acetamide, a food contaminant, has been shown to induce hepatocellular tumors in rats. However, the mode of action underlying acetamide-induced hepatocarcinogenesis remains unclear. In the current study, we aimed to examine the possible involvement of *in vivo* mutagenicity in hepatocarcinogenesis of acetamide and evaluate its toxicological profile using a comprehensive medium-term toxicity study in *gpt* delta rats. Six-week-old male F344 *gpt* delta rats were given a basal diet containing 0%, 0.625%, 1.25%, or 2.5% acetamide for 13 weeks. In general toxicologic assessment, hepatotoxic parameters in serum, such as aspartate aminotransferase and alanine aminotransferase were significantly changed at the 1.25% group and higher. Histopathological examination of the liver revealed that various changes related to

hepatic injury were observed at the 1.25% group and higher. Interestingly, Feulgen-positive cytoplasmic inclusion was frequently observed in hepatocytes in these groups. In the hematopoietic system, red blood cell parameters in plasma, such as mean corpuscular volume and mean corpuscular hemoglobin were significantly changed at the 1.25% group and higher, and decrease of erythroblast in the spleen was observed histopathologically in the 2.5% group. Thus, the no-observed-adverse-effect level of acetamide in this study was 0.625% (equivalent to 394 mg/kg body weight/day). *In vivo* mutation assays showed that acetamide induced no changes in *gpt* and *red/gam* gene mutant frequencies, even at the carcinogenic target site. In contrast, Ki67-positive hepatocytes were increased significantly at carcinogenic doses. Therefore, these results suggested that cell proliferation activity, but not mutagenicity, played crucial roles in acetamide-induced hepatocarcinogenesis in rats.

Keywords: *gpt* delta rat, acetamide, *in vivo* mutagenicity

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Honma M, Kitazawa A, Kasamatsu T, Sugiyama K: Screening for Ames mutagenicity of food flavor chemicals by (quantitative) structure-activity relationship.

Genes Environ. 2020;42:32. doi: 10.1186/s41021-020-00171-1

Background: (Quantitative) Structure-Activity Relationship ((Q)SAR) is a promising approach to predict the potential adverse effects of chemicals based on their structure without performing toxicological studies. We evaluate the mutagenicity of food flavor chemicals by (Q) SAR tools, identify potentially mutagenic chemicals, and verify their mutagenicity by actual Ames test.

Results: The Ames mutagenicity of 3942 food flavor chemicals was predicted using two (Q)SAR) tools, DEREK Nexus and CASE Ultra. Three thousand five hundred seventy-five chemicals (91%) were judged to be negative in both (Q) SAR tools, and 75 chemicals (2%) were predicted to be positive in both (Q) SAR tools. When the Ames test was conducted on ten of these positive chemicals, nine showed positive results.

Conclusion: The (Q) SAR method can be used for screening the mutagenicity of food flavors.

Keywords: (quantitative) structure-activity relationship ((Q)SAR), Ames test, food flavors

Van Bossuyt M[*1,2] Raitano G[*3], Honma M, Van Hoeck E[*1], Vanhaecke T[*2], Rogiers V[*2], Mertens B[*1], Benfenati E[*3]: New QSAR models to predict chromosome damaging potential based on the *in vivo* micronucleus test.

Toxicol Lett. 2020;329:80. doi: 10.1016/j.toxlet.2020.04.016

A large number of computer-based prediction methods to determine the potential of chemicals to induce mutations at the gene level has been developed over the last decades. Conversely, only few such methods are currently available to predict potential structural and numerical chromosome aberrations. Even fewer of these are based on the preferred testing method for this endpoint, *i.e.* the micronucleus test. For the present work, *in vivo* micronucleus test results of 718 structurally diverse compounds were collected and applied for the construction of new models by means of the freely available SARpy *in silico* model building software. Multiple QSAR models were created using parameter variation and manual verification of (non-) alerting structures. To this extent, the original set of 718 compounds was split into a training (80%) and a test (20%) set. SARpy was applied on the training set to automatically extract sets of rules by generating and selecting substructures based on their prediction performance whereas the test set was used to evaluate model performance. Five different splits were made randomly, each of which had a similar balance between positive and negative substances compared to the full dataset. All generated models were characterised by an overall better performance than existing free and commercial models for the same endpoint, while demonstrating high coverage.

Keywords: chromosome damage, *in vivo* micronucleus, QSAR

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Masumura K, Yatagai F[*1], Ochiai M[*2], Nakagama H[*2], Nohmi T: Effects of the *scid* mutation on X-ray-induced deletions in the brain and spleen of *gpt* delta

mice.

Genes Environ. 2020;42:19. doi: 10.1186/s41021-020-00158-y

Background: DNA-dependent protein kinase (DNA-PK), consisting of a Ku heterodimer (Ku70/80) and a large catalytic subunit (DNA-PKcs), plays an important role in the repair of DNA double-strand breaks via nonhomologous end-joining (NHEJ) in mammalian cells. Severe combined immunodeficient (*scid*) mice carry a mutation in the gene encoding DNA-PKcs and are sensitive to ionizing radiation. To examine the roles of DNA-PKcs in the generation of deletion mutations *in vivo*, we crossed *scid* mice with *gpt* delta transgenic mice for detecting mutations.

Results: The *scid* and wild-type (WT) *gpt* delta transgenic mice were irradiated with a single X-ray dose of 10 Gy, and Spi^- mutant frequencies (MFs) were determined in the brain and spleen 2 days after irradiation. Irradiation with X-rays significantly enhanced Spi^- MF in both organs in the *scid* and WT mice. The MFs in the brain of irradiated *scid* mice were significantly lower than those in WT mice, i.e., $2.9 \pm 1.0 \times 10^{-6}$ versus $5.0 \pm 1.1 \times 10^{-6}$ ($P < 0.001$), respectively. In the spleen, however, both mouse strains exhibited similar MFs, i.e., $4.1 \pm 1.8 \times 10^{-6}$ versus $4.8 \pm 1.4 \times 10^{-6}$. Unirradiated *scid* and WT mice did not exhibit significant differences in MFs in either organ.

Conclusions: DNA-PKcs is unessential for the induction of deletion mutations in the spleen, while it plays a role in this in the brain. Therefore, the contribution of DNA-PKcs to NHEJ may be organ-specific.

Keywords: *scid* mice, deletion, X-irradiation

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Chen R*1, You X*1, Cao Y*1, Masumura K, Ando T, Hamada S*2, Horibata K, Wan J*1, Xi J*1, Zhang X*1, Honma M, Luan Y.*1: Benchmark dose analysis of multiple genotoxicity endpoints in *gpt* delta mice exposed to aristolochic acid I.

Mutagenesis. 2020;geaa034. doi: 10.1093/mutage/geaa034

As the carcinogenic risk of herbs containing aristolochic acids (AAs) is a global health issue, quantitative evaluation of toxicity is needed for the regulatory decision-making and risk assessment of AAs. In this study, we selected AA I (AAI), the most abundant and representative compound in AAs, to treat transgenic *gpt* delta mice at six gradient doses ranging from 0.125 to 4 mg/kg/day for 28 days. AAI-DNA adduct frequencies and *gpt* gene mutation frequencies (MFs) in the kidney, as well as *Pig-a* gene MFs and micronucleated reticulocytes (MN-RETs) frequencies in peripheral blood, were monitored. The dose-response (DR) relationship data for these *in vivo* genotoxicity endpoints were quantitatively evaluated using an advanced benchmark dose (BMD) approach with different critical effect sizes (CESs; i.e., BMD_5, BMD_{10}, BMD_{50} and BMD_{100}). The results showed that the AAI-DNA adduct frequencies, *gpt* MFs and the MN-RETs presented good DR relationship to the administrated doses, and the corresponding $BMDL_{100}$ (the lower 90% confidence interval of the BMD_{100}) values were 0.017, 0.509 and 3.9 mg/kg/day, respectively. No positive responses were observed in the *Pig-a* MFs due to bone marrow suppression caused by AAI. Overall, we quantitatively evaluated the genotoxicity of AAI at low doses for multiple endpoints for the first time. Comparisons of BMD_{100} values across different endpoints provide a basis for the risk assessment and regulatory decision-making of AAs and are also valuable for understanding the genotoxicity mechanism of AAs.

Keywords: aristolochic acids, benchmark dose, genotoxicity

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Hagio S*, Tsuji N*, Furukawa S*, Takeuchi K*, Hayashi S*, Kuroda Y*, Honma M, Masumura K: Effect of sampling time on somatic and germ cell mutations induced by acrylamide in *gpt* delta mice.

Genes Environ. 2021;43:4. doi: 10.1186/s41021-021-00175-5

Background: Acrylamide (AA) is a rodent carcinogen and classified by the IARC into Group 2A (probable human carcinogen). AA has been reported to induce mutations in transgenic rodent gene mutation assays (TGR assays), the extent of which is presumed to depend on exposure length and the

duration of expression after exposure. In particular, it is not clear in germ cells. To investigate mutagenicity with AA in somatic and germ cells at different sampling times, we conducted TGR assays using *gpt* delta transgenic mice.

Results: The male *gpt* delta mice at 8 weeks of age were treated with AA at 7.5, 15 and 30 mg/kg/day by gavage for 28 days. Peripheral blood was sampled on the last day of the treatment for micronucleus tests and tissues were sampled for gene mutation assays at day 31 and day 77, those being 3 and 49 days after the final treatment (28 + 3d and 28 + 49d), respectively. Another group of mice was treated with *N*-ethyl-*N*-nitrosourea (ENU) at 50 mg/kg/day by intraperitoneal administration for 5 consecutive days and tissues were sampled at the day 31 and day 77 (5 + 26d and 5 + 72d). Frequencies of micronucleated erythrocytes in the peripheral blood significantly increased at AA doses of 15 and 30 mg/kg/day. Two- to three-fold increases in *gpt* mutation frequencies (MFs) compared to vehicle control were observed in the testes and lung treated with 30 mg/kg/day of AA at both sampling time. In the sperm, the *gpt* MFs and G:C to T:A transversions were significantly increased at 28 + 3d, but not at 28 + 49d. ENU induced *gpt* mutations in these tissues were examined at both 5 + 26d and 5 + 72d. A higher mutant frequency in the ENU-treated sperm was observed at 5 + 72d than that at 5 + 26d.

Conclusions: The *gpt* MFs in the testes, sperm and lung of the AA-treated mice were determined and compared between different sampling times (3 days or 49 days following 28 day-treatment). These results suggest that spermatogonial stem cells are less sensitive to AA mutagenicity under the experimental condition. Prolonged expression time after exposure to AA to detect mutagenicity may be effective in somatic cells but not in germ cells.

Keywords: acrylamide, germ cell, *gpt* delta mouse

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Gajewicz-Skretna A*1, Gromelski M*1, Wyrzykowska E*1, Furuhama A, Yamamoto H*2, Suzuki N*2: Aquatic toxicity (Pre)screening strategy for structurally diverse chemicals: global or local classification tree models?

Ecotoxicol Environ Saf. 2021;208:111738. doi: 10.1016/j.ecoenv.2020.111738

With an ever-increasing number of synthetic chemicals being manufactured, it is unrealistic to expect that they will all be subjected to comprehensive and effective risk assessment. A shift from conventional animal testing to computer-aided methods is therefore an important step towards advancing the environmental risk assessments of chemicals. The aims of this study are two-fold: firstly, it examines the relationships between structural and physicochemical features of a diverse set of organic chemicals, and their acute aquatic toxicity towards *Daphnia magna* and *Oryzias latipes* using a classification tree approach. Secondly, it compares the efficiency and accuracy of the predictions of two modeling schemes: local models that are inherently restricted to a smaller subset of structurally-related substances, and a global model that covers a wider chemical space and a number of modes of toxic action. The classification tree-based models differentiate the organic chemicals into either 'highly toxic' or 'low to non-toxic' classes, based on internal and external validation criteria. These mechanistically-driven models, which demonstrate good performance, reveal that the key factors driving acute aquatic toxicity are lipophilicity, electrophilic reactivity, molecular polarizability and size. A comparative analysis of the performance of the two modeling schemes indicates that the local models, trained on homogeneous data sets, are less error prone, and therefore superior to the global model. Although the global models showed worse performance metrics compared to the local ones, their applicability domain is much wider, thereby significantly increasing their usefulness in practical applications for regulatory purposes. This demonstrates their advantage over local models and shows they are an invaluable tool for modeling heterogeneous chemical data sets.

Keywords: acute aquatic toxicity, global/local models, hierarchical clustering analysis

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Yasui M, Fukuda T*1, Ukai A, Maniwa J*2, Imamura T*3, Hashizume T*4, Yamamoto H*4, Shibuya K*4, Narumi K*5, Fujiishi Y*5, Okada E*5, Fujishima S*6, Yamamoto M*7, Otani N*7, Nakamura M*1,

Nishimura R[*1], Ueda M[*8], Mishima M[*9], Matsuzaki K[*9], Takeiri A[*9], Tanaka K[*9], Okada Y[*10], Nakagawa M[*11], Hamada S[*1], Kajiwara A[*11], Honda H[*12], Adachi J[*13], Misaki K[*14], Ogawa K, Honma M: Weight of evidence approach using a *TK* gene mutation assay with human TK6 cells for follow-up of positive results in Ames tests: a collaborative study by MMS/JEMS.

Genes Environ. 2021;43:7. doi: 10.1186/s41021-021-00179-1

Background: Conflicting results between bacterial mutagenicity tests (the Ames test) and mammalian carcinogenicity tests might be due to species differences in metabolism, genome structure, and DNA repair systems. Mutagenicity assays using human cells are thought to be an advantage as follow-up studies for positive results in Ames tests. In this collaborative study, a thymidine kinase gene mutation study (TK6 assay) using human lymphoblastoid TK6 cells, established in OECD TG490, was used to examine 10 chemicals that have conflicting results in mutagenicity studies (a positive Ames test and a negative result in rodent carcinogenicity studies).

Results: Two of 10 test substances were negative in the overall judgment (20% effective as a follow-up test). Three of these eight positive substances were negative after the short-term treatment and positive after the 24 h treatment, despite identical treatment conditions without S9. A toxicoproteomic analysis of TK6 cells treated with 4-nitroanthranilic acid was thus used to aid the interpretation of the test results. This analysis using differentially expressed proteins after the 24 h treatment indicated that in vitro specific oxidative stress is involved in false positive response in the TK6 assay.

Conclusions: The usefulness of the TK6 assay, by current methods that have not been combined with new technologies such as proteomics, was found to be limited as a follow-up test, although it still may help to reduce some false positive results (20%) in Ames tests. Thus, the combination analysis with toxicoproteomics may be useful for interpreting false positive results raised by 24 h specific reactions in the assay, resulting in the more reduction (> 20%) of false positives in Ames test.

Keywords: Ames test, TK6 assay, toxicoproteomics

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Takeishi A[*1], Kogashi H[*1], Odagiri M[*1], Sasanuma H[*2], Takeda S[*2], Yasui M, Honma M, Suzuki T[*3], Kamiya H[*3], Sugasawa K[*4], Ura K[*1], Sassa A[*1]: Tyrosyl-DNA phosphodiesterases are involved in mutagenic events at a ribonucleotide embedded into DNA in human cells.

PLoS One. 2020;15:e0244790. doi: 10.1371/journal.pone.0244790

Ribonucleoside triphosphates are often incorporated into genomic DNA during DNA replication. The accumulation of unrepaired ribonucleotides is associated with genomic instability, which is mediated by DNA topoisomerase 1 (Top1) processing of embedded ribonucleotides. The cleavage initiated by Top1 at the site of a ribonucleotide leads to the formation of a Top1-DNA cleavage complex (Top1cc), occasionally resulting in a DNA double-strand break (DSB). In humans, tyrosyl-DNA phosphodiesterases (TDPs) are essential repair enzymes that resolve the trapped Top1cc followed by downstream repair factors. However, there is limited cellular evidence of the involvement of TDPs in the processing of incorporated ribonucleotides in mammals. We assessed the role of TDPs in mutagenesis induced by a single ribonucleotide embedded into DNA. A *supF* shuttle vector site-specifically containing a single riboguanosine (rG) was introduced into the human lymphoblastoid TK6 cell line and its *TDP1*-, *TDP2*-, and *TDP1*/*TDP2*-deficient derivatives. *TDP1* and *TDP2* insufficiency remarkably decreased the mutant frequency caused by an embedded rG. The ratio

of large deletion mutations induced by rG was also substantially lower in *TDP1*/*TDP2*-deficient cells than wild-type cells. Furthermore, the disruption of TDPs reduced the length of rG-mediated large deletion mutations. The recovery ratio of the propagated plasmid was also increased in *TDP1*/*TDP2*-deficient cells after the transfection of the shuttle vector containing rG. The results suggest that TDPs-mediated ribonucleotide processing cascade leads to unfavorable consequences, whereas in the absence of these repair factors, a more error-free processing pathway might function to suppress the ribonucleotide-induced mutagenesis. Furthermore, base substitution mutations at sites outside the position of rG were detected in the *supF* gene via a TDPs-independent mechanism. Overall, we provide new insights into the mechanism of mutagenesis induced by an embedded ribonucleotide in mammalian cells, which may lead to the fatal phenotype in the ribonucleotide excision repair deficiency.

Keywords: ribonucleotide, DNA topoisomerase 1, tyrosyl-DNA phosphodiesterase

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Ibrahim MA*1, Yasui M, Saha LK*1, 2, Sasanuma H*1, Honma M, Takeda S*1: Enhancing the sensitivity of the thymidine kinase assay by using DNA repair-deficient human TK6 cells.

Environ Mol Mutagen. 2020;61:602-610. doi: 10.1002/em.22371

The OECD guidelines define the bioassays of identifying mutagenic chemicals, including the thymidine kinase (*TK*) assay, which specifically detects the mutations that inactivate the *TK* gene in the human TK6 lymphoid line. However, the sensitivity of this assay is limited because it detects mutations occurring only in the *TK* gene but not any other genes. Moreover, the limited sensitivity of the conventional *TK* assay is caused by the usage of DNA repair-proficient *wild-type* cells, which are capable of accurately repairing DNA damage induced by chemicals. Mutagenic chemicals produce a variety of DNA lesions, including base lesions, sugar damage, crosslinks, and strand breaks. Base damage causes point mutations and is repaired by the base excision repair (BER) and nucleotide excision repair (NER) pathways. To increase the sensitivity of TK assay, we simultaneously disrupted two genes encoding *XRCC1*, an important BER factor, and XPA, which is essential for NER, generating $XRCC1^{-/-}/XPA^{-/-}$ cells from TK6 cells. We measured the mutation frequency induced by four typical mutagenic agents, methyl methane sulfonate (MMS), cis-diamminedichloroplatinum(II) (cisplatin, CDDP), mitomycin-C (MMC), and cyclophosphamide (CP) by the conventional *TK* assay using *wild-type* TK6 cells and also by the *TK* assay using $XRCC1^{-/-}/XPA^{-/-}$ cells. The usage of $XRCC1^{-/-}/XPA^{-/-}$ cells increased the sensitivity of detecting the mutagenicity by 8.6 times for MMC, 8.5 times for CDDP, and 2.6 times for MMS in comparison with the conventional *TK* assay. In conclusion, the usage of $XRCC1^{-/-}/XPA^{-/-}$ cells will significantly improve *TK* assay.

Keywords: DNA-damaging agent, OECD guideline, TK6 cells

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Tanabe S, Quader S*1, Ono R, Cabral H*2, Aoyagi K*3, Hirose A, Yokozaki H*4, Sasaki H*3: Molecular Network Profiling in Intestinal- and Diffuse-Type Gastric Cancer

Cancers. 2020;12:3833. doi: 10.3390/cancers12123833

Epithelial-mesenchymal transition (EMT) plays an important role in the acquisition of cancer stem cell (CSC) feature and drug resistance, which are the main hallmarks of cancer malignancy. Although previous findings have shown that several signaling pathways are activated in cancer progression, the precise mechanism of signaling pathways in EMT and CSCs are not fully understood. In this study, we focused on the intestinal and diffuse-type gastric cancer (GC) and analyzed the gene expression of public RNAseq data to understand the molecular pathway regulation in different subtypes of gastric cancer. Network pathway analysis was performed by Ingenuity Pathway Analysis (IPA). A total of 2815 probe set IDs were significantly different between intestinal- and

diffuse-type GC data in cBioPortal Cancer Genomics. Our analysis uncovered 10 genes including male-specific lethal 3 homolog (Drosophila) pseudogene 1 (MSL3P1), CDC28 protein kinase regulatory subunit 1B (CKS1B), DEAD-box helicase 27 (DDX27), golgi to ER traffic protein 4 (GET4), chromosome segregation 1 like (CSE1L), translocase of outer mitochondrial membrane 34 (TOMM34), YTH N6-methyladenosine RNA binding protein 1 (YTHDF1), ribonucleic acid export 1 (RAE1), par-6 family cell polarity regulator beta (PARD6B), and MRG domain binding protein (MRGBP), which have differences in gene expression between intestinal- and diffuse-type GC. A total of 463 direct relationships with three molecules (MYC, NTRK1, UBE2M) were found in the biomarker-filtered network generated by network pathway analysis. The networks and features in intestinal- and diffuse-type GC have been investigated and profiled in bioinformatics. Our results revealed the signaling pathway networks in intestinal- and diffuse-type GC, bringing new light for the elucidation of drug resistance mechanisms in CSCs.

Keywords: cancer stem cell, epithelial-mesenchymal transition, molecular network

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Kawashima A, Inoue K, Yoshizaki Y, Ushida K, Kai K, Suzuki H, Takano M*1, Fujii S*2, Yabe K*2, Matsumoto M, Yamada T and Hirose A: Combined repeated-dose and reproductive/developmental oral toxicity of 3-methylpentane, isooctane, and isononane in rats.

Fundam Toxicol Sci. 2020;7:259-279. doi: 10.2131/fts.7.259

3-Methylpentane, isooctane, and isononane are acyclic branched saturated hydrocarbons with carbon numbers C6, C8, and C9, respectively. To assess human risk, we conducted a combined repeated-dose and reproductive/developmental oral toxicity studies in rats. [Organization for Economic Co-operation and Development (OECD) Test Guideline 422]. Each hydrocarbon was administered by gavage to rats at three doses (plus a control group). All three chemicals targeted the liver and kidney. An increase in liver weight without hepatic injury was observed as the adaptive response to the chemical treatments. Males treated by each chemical showed α2u-globulin nephropathy, which is a rat-specific finding that bears no human relevance. Reproduction/developmental toxicity parameters showed no treatment-related effects in parents or offspring at any dose for the three chemicals, except for the retardation of offspring bodyweight development which may be a secondary effect of a maternal systemic condition or direct effect on offspring in isononane. No observed adverse effect levels (NOAELs) of repeated toxicity in either sex were determined to be 300 mg/kg/day for 3-methylpentane, 100 mg/kg/day for isooctane, and 250 mg/kg/day for isononane. NOAELs of reproductive/developmental toxicity in parents and offspring were determined to be 1000 mg/kg/day for 3-methylpentane, and 300 mg/kg/day for isooctane. For isononane, NOAELs were determined to be 1000 mg/kg/day for reproduction, and 250 mg/kg/day for offspring development. These results provide new toxicological information and support the category assessment of published reports that evaluate the acyclic branched saturated hydrocarbons as low-toxicity substances.

Keywords: 3-Methylpentane (CAS No. 96-14-0), Isooctane (CAS No. 26635-64-3), Isononane (CAS No. 34464-40-9)

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Yamazoe Y*1,2, Yamada T, Nagata K*3: Prediction and Characterization of CYP3A4-mediated metabolisms of azole fungicides: an application of the fused-grid template system.

Food Saf (*Tokyo*). 2020;8(2):34-51. doi: 10.14252/foodsafetyfscj.D-20-00010. eCollection 2020 Jun.

Human CYP3A4 is involved in metabolisms of diverse hydrophobic chemicals. Using the data of therapeutic azole fungicides known to interact with CYP3A4, applicability of CYP3A4 Template system was first confirmed to reconstitute faithfully the interaction on Template. More than twenty numbers of pesticide azoles were then applied to the Template

system. All the azole stereo-isomers applied, except for talarozole, interacted through nitrogen atoms of triazole or imidazole parts and sat stably for inhibitions through fulfilling three-essential interactions. For their CYP3A4-mediated oxidations, clear distinctions were suggested among the enantiomers and diastereomers of azole pesticides on Templates. Thus, the stereoisomers would have their-own regio- and stereo-selective profiles of the metabolisms. A combined metabolic profile of each azole obtained with CYP3A4 Template system, however, resembled with the reported profile of the *in vivo* metabolism in rats. These results suggest the major roles of CYP3A forms on the metabolisms of most of azole pesticides in both rats and humans. Free triazole is a metabolite of azole fungicides having a methylene-spacer between triazole and the rest of the main structures in experimental animals and humans. During the simulation experiments, a placement for the oxidation of a methylene spacer between the triazole and main carbon-skeleton was found to be available throughout the azole fungicides tested on Template. The occurrence of this reaction to lead to triazole-release is thus discussed in relation to the possible involvement of CYP3A forms.

Keywords: CYP3A4 inhibition and metabolism, fungicide

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山田隆志：IATAの実践および毒性データベースとin silicoツールの利用から学んだ知見.
フロンティア. 2020;2(3):133-139.

Hazard assessment of a huge number of chemical substances without toxicity test data has become a major issue. There is a strong demand for improving the Integrated Approaches to Testing and Assessment (IATA)-based read-across for filling data gaps of systemic toxicity endpoints. Herein, I demonstrate how read-across is undertaken by introducing the case study which I developed for hazard assessment of a particular class of chemical substances of high concern to human health. The case study was reviewed by the experts of OECD IATA Case Studies Project. Sharing the experience of development and the review of read-across cases is valuable for guiding principles for different read-across decision contexts and further acceptance of the predictions in the future. Moreover, importance of read-across resources including the toxicity database and *in silico* prediction tool are discussed based on my experience of their development and use.

Keywords：毒性データベース，リードアクロス，行政受入

Rovida C*1, Escher SE*2, Herzler M*3, Hougaard Bennekou S*4, Kamp H*5, Kroese, DE*6, Maslankiewicz L*7, Moné MJ*8, Patlewicz G*9, Sipes N*10, van Aerts L*11, White A*12, Yamada T, van de Water B*8: NAM-supported read-across: From case studies to regulatory guidance in safety assessment. *ALTEX*. 2021;38(1):140-150. doi: 10.14573/altex.2010062

The use of new approach methodologies (NAMs) in support of read-across (RAx) approaches for regulatory purposes is a main goal of the EU-ToxRisk project. To bring this forward, EU-ToxRisk partners convened a workshop in close collaboration with regulatory representatives from key organizations including European regulatory agencies, such as the European Chemicals Agency (ECHA) and the European Food Safety Authority (EFSA), as well as the Scientific Committee on Consumer Safety (SCCS), national agencies from several European countries, Japan, Canada and the USA, as well as the Organisation for Economic Cooperation and Development (OECD). More than a hundred people actively participated in the discussions, bringing together diverse viewpoints across academia, regulators and industry. The discussion was organized starting from five practical cases of RAx applied to specific problems that offered the opportunity to consider real examples. There was general consensus that NAMs can improve confidence in RAx, in particular in defining category boundaries as well as characterizing the similarities/dissimilarities between

source and target substances. In addition to describing dynamics, NAMs can be helpful in terms of kinetics and metabolism that may play an important role in the demonstration of similarity or dissimilarity among the members of a category. NAMs were also noted as effective in providing quantitative data correlated with traditional no observed adverse effect levels (NOAELs) used in risk assessment, while reducing the uncertainty on the final conclusion. An interesting point of view was the advice on calibrating the number of new tests that should be carefully selected, avoiding the allure of "the more, the better". Unfortunately, yet unsurprisingly, there was no single approach befitting every case, requiring careful analysis delineating the optimal approach. Expert analysis and assessment of each specific case is still an important step in the process.

Keywords: read across, NAMs, regulatory evaluation

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Yamazoe Y*1, 2, Yamada T, Hirose A, Murayama N*3: Deciphering key interactions of ligands with CYP3A4-Template system.

Food Saf (*Tokyo*). 2021;9(1):10-21. doi: 10.14252/foodsafetyfscj.D-20-00023

Cytochrome P450 (CYP)-mediated metabolisms are often associated with biological and toxicological events of chemicals. A major hepatic enzyme, CYP3A4, showed clear distinctions on their catalyses even among ligands having resemble structures. To better understand mechanisms of their distinct catalyses, possible associations of ligand interactions at specific parts of CYP3A4 residues were investigated using CYP3A4-Template system developed (DMPK 2019 and 2020). A placement was available selectively for CYP3A4-mediated R-thalidomide 5-oxidation on Template, but not for the 5'-oxidation and the S-isomer oxidations. Similar placements were generated for pomalidomide (4-amino-thalidomide), but not for a poor ligand, lenalidomide (3-deoxy-pomalidomide). The latter ligand took placements lacking IJK-Interaction or sticking the 4-amino part beyond the facial-side wall on Template. A placement was available for the tert-butyl oxidation of terfenadine, but not for an analog, ebastine. Their interactions with upper-Cavity-2 residue were expected to differ at their sites of oxygen substituents. Some phenolic antioxidants behave distinctly toward biological oxidations *in vitro* and *in vivo*. Butylated hydroxytoluene is oxidized to the peroxy-derivative in vitro, but solely to the oxidized metabolites at the benzyl and tert-butyl methyl positions *in vivo*. Involvement of CYP3A4 were suggested for all the three reactions from the placements on Template. Tocopherols were also applied on Template for the oxidations for chroman and side-chain terminals. The primary placement was suggested to undergo the futile-recycling through formation of the peroxide intermediate subsequently to lead the substantial lack of the CYP3A4-mediated oxidation. These data suggest the effectiveness of CYP3A4-Template assessment to understand the causal basis of poor oxidations and also to verify the *in vivo* contribution of CYP3A4-mediated peroxidative reactions.

Keywords: thalidomide, lenalidomide, ebastine

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Yamada T, Kurimoto M, Hirose A, Yang C*1,2, Rathman J*1,2: Development of a new threshold of toxicological concern database of non-cancer toxicity endpoints for industrial chemicals.
Front. Toxicol. 202;3:1-9. doi: 10.3389/ftox.2021.626543

In cases where chemical-specific toxicity data are absent or limited, the threshold of toxicological concern (TTC) offers an alternative to assess human exposure below which "there would be no appreciable risk to human health." The application of TTC to non-cancer systemic endpoints has been pursued for decades using a chemical classification and Point of Departure (POD). This study presents a new POD dataset of oral subacute/subchronic toxicity studies in rats for 656 industrial chemicals retrieved from the Hazard Evaluation Support System (HESS) Integrated Platform, which contains hundreds of reliable repeated-dose toxicity test data of industrial chemicals under the Chemical Substances of Control Law in Japan. The HESS TTC dataset was found to have less duplication with substances in other reported TTC datasets. Each chemical was classified into a Cramer Class, with 68, 3, and 29% of these 656 chemicals distributed in Classes III, II, and I, respectively. For each Cramer Class, a provisional Tolerable Daily Intake (TDI) was derived from the 5th percentile of the lognormal distribution of PODs. The TDIs were 1.9 and 30 μg/kg bw/day for Classes III and I, respectively. The TDI for Cramer Class II could not be determined due to insufficient sample size. This work complements previous studies of the TTC approach and increases the confidence of the thresholds for non-cancer endpoints by including unique chemical structures. This new TTC dataset is publicly available and can be merged with existing databases to improve the TTC approach.
Keywords: TTC database, industrial chemicals, non-cancer endpoint

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Mizoi K*1, Hosono M*1, Kojima H, Ogihara T*2: Establishment of a primary human hepatocyte spheroid system for evaluating metabolic toxicity using dacarbazine under conditions of CYP1A2 induction.
Drug Metab Pharmacokinet. 2020,35(2):201-206. doi: 10.1016/j.dmpk.2019.11.002

Some drugs induce cytochrome P450s (CYPs), and thus may cause increased metabolic toxicity from concomitantly administered agents. Hence, we need a means of evaluating the potential of compounds to cause drug-induced liver injury (DILI) under conditions where inducers of CYP1A2 are present. Here, we present a system for evaluating CYP1A2-mediated metabolic toxicity using three-dimensional (3D) cultures of primary human hepatocyte spheroids treated with the CYP1A2 inducer omeprazole (OPZ). As a test substrate, we employed dacarbazine (DTIC), which causes toxicity during the metabolic process. We measured cell viability, CYP1A2 mRNA expression level and metabolism of DTIC, as well as several markers of hepatic function, i.e. albumin secretion, urea secretion, and aspartate aminotransferase (AST) leakage. Markers of hepatic function were significantly decreased by addition of OPZ and DTIC even under conditions where the cell viability was largely unchanged. This experimental system sensitively detected CYP1A2-mediated metabolic toxicity. Therefore, the developed system should be helpful for evaluating the potential of compounds to cause DILI under conditions where inducers of CYP1A2 are present.
Keywords: cytochrome P450s (CYPs), drug-induced liver injury (DILI), primary human hepatocyte spheroid system

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Mizoi K*1, Arakawa H*1,2, Yano K*1,3, Koyama S*1, Kojima H, Ogihara T*1,4: Utility of Three-

Dimensional Cultures of Primary Human Hepatocytes (Spheroids) as Pharmacokinetic Models.

JBiomedicines. 2020;8(10):374. doi: 10.3390/biomedicines8100374

This paper reviews the usefulness, current status, and potential of primary human hepatocytes (PHHs) in three-dimensional (3D) cultures, also known as spheroids, in the field of pharmacokinetics (PK). Predicting PK and toxicity means pharmaceutical research can be conducted more efficiently. Various in vitro test systems using human hepatocytes have been proposed as tools to detect hepatic toxicity at an early stage in the drug development process. However, such evaluation requires long-term, low-level exposure to the test compound, and conventional screening systems such as PHHs in planar (2D) culture, in which the cells can only survive for a few days, are unsuitable for this purpose. In contrast, spheroids consisting of PHH are reported to retain the functional characteristics of human liver for at least 35 days. Here, we introduce a fundamental PK and toxicity assessment model of PHH spheroids and describe their applications for assessing species-specific metabolism, enzyme induction, and toxicity, focusing on our own work in these areas. The studies outlined in this paper may provide important information for pharmaceutical companies to reduce termination of development of drug candidates.

Keywords: cytochrome P450s (CYPs), drug-induced liver injury (DILI), primary human hepatocyte spheroid system

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Kato Y[*1], Yamamoto N[*2], Hiramatsu N[*3], Sato A[*1], Kojima H: Inhouse Fabrication of a Reconstructed Human Corneal Epithelium Model for Use in Testing for Eye Irritation Potential.

Applied in Vitro Toxicology. 2020;6(3), doi: 10.1089/aivt.2020.0003

In the last decade, a variety of in vitro eye irritation test (EIT) methods have been developed and validated as an alternative to animal testing to assess the ocular toxicity of chemicals. Among these in vitro test methods, that using reconstructed human corneal epithelium (RhCE) is considered to be most useful, but RhCE is expensive and cannot be purchased at any time in Japan. Thus, we undertook this study to establish a method for in house fabrication of RhCE using the immortalized human corneal epithelial cell lines.

Keywords: eye irritation test (EIT), human corneal epithelium (RhCE), immortalized human corneal epithelial cell lines

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Imamura M[*1], Wanibuchi S[*1], Yamamoto Y[*1], Kojima H, Ono A[*2], Kasahara T[*1], Fujita M[*1]: Improving predictive capacity of the Amino acid Derivative Reactivity Assay test method for skin sensitization potential with an optimal molar concentration of test chemical solution.

J Appl Toxicol. 2021;41(2):303-329. doi: 10.1002/jat.4082

The Amino acid Derivative Reactivity Assay (ADRA) is a convenient and effective *in chemico* test method for assessing covalent binding of test chemicals with protein-derived nucleophilic reagents as a means of predicting skin sensitization potential. Although the original molar-concentration approach to ADRA testing was not suitable for testing multiconstituent substances of an unknown composition, a weight-concentration approach that is suitable for such substances was developed, which also led to the realization that test chemical solutions prepared to molar concentrations higher than the original 1 mM would reduce false negative results as well as enhance predictive capacity. The present study determined an optimal molar-concentration that achieves even higher predictive capacity than the original ADRA. Eight chemicals that were false negatives when tested with 1 mM test chemical solutions were retested with

test chemical solutions between 2 and 5 mM, which showed 4 mM to be the optimal molar-concentration for ADRA testing. When 82 chemicals used in the original development were retested with 4 mM test chemical solutions, false negative results were reduced by four. When an additional 85 chemicals used to evaluate the weight-concentration approach to ADRA were retested, the results essentially replicated those obtained with 0.5 mg/ml test chemical solutions and gave 10 fewer false negatives than original ADRA with 1 mM solutions. A comparison of these results for 136 chemicals showed that ADRA testing with 4 mM solutions achieved a four percentage point improvement in accuracy over original ADRA and a two percentage point improvement over DPRA testing.

Keywords: Amino acid Derivative Reactivity Assay (ADRA), *in chemico*, skin sensitization

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BACKGROUND: Multi-walled carbon nanotubes can be divided into two general subtypes: tangled and straight. MWCNT-N (60 nm in diameter) and MWCNT-7 (80-90 nm in diameter) are straight-type MWCNTs, and similarly to asbestos, both are carcinogenic to the lung and pleura when administered to rats via the airway. Injection of straight-type MWCNTs into the peritoneal cavity also induces the development of mesothelioma, however, injection of tangled-type MWCNTs into the peritoneal cavity does not induce carcinogenesis. To investigate these effects in the lung we conducted a 2-year comparative study of the potential carcinogenicities of a straight-type MWCNT, MWCNT-A (approximately 150 nm in diameter), and a tangled-type MWCNT, MWCNT-B (7.4 nm in diameter) after administration into the rat lung. Crocidolite asbestos was used as the reference material, and rats administered vehicle were used as the controls. Test materials were administered by intra-Tracheal Intra-Pulmonary Spraying (TIPS) once a week over a 7 week period (8 administrations from day 1 to day 50), followed by a 2-year observation period without further treatment. Rats were administered total doses of 0.5 or 1.0 mg MWCNT-A and MWCNT-B or 1.0 mg asbestos.

RESULTS: There was no difference in survival between any of the groups. The rats administered MWCNT-A or asbestos did not have a significant increase in bronchiolo-alveolar hyperplasia or tumors in the lung. However, the rats administered MWCNT-B did have significantly elevated incidences of bronchiolo-alveolar hyperplasia and tumors in the lung: the incidence of bronchiolo-alveolar hyperplasia was 0/20, 6/20, and 9/20 in the vehicle, 0.5 mg MWCNT-B, and 1.0 mg MWCNT-B groups, respectively, and the incidence of adenoma and adenocarcinoma combined was 1/19, 5/20, and 7/20 in the vehicle, 0.5 mg MWCNT-B, and 1.0 mg MWCNT-B groups, respectively. Malignant pleural mesothelioma was not induced in any of the groups.

CONCLUSIONS: The results of this initial study indicate that tangled-type MWCNT-B is carcinogenic to the rat lung when administered via the airway, and that straight-type MWCNT-A did not have higher carcinogenic potential in the rat lung than tangled-type MWCNT-B.

Keywords: carcinogenicity, intratracheal, MWCNT

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Yoshii K*1, Nishiura H*1, Inoue K, Yamaguchi T*2, Hirose A: Simulation-based assessment of model selection criteria during the application of benchmark dose method to quantal response data. *Theor Biol Med Model*. 2020; 7(1):13. doi: 10.1186/s12976-020-00131-w.

Background: To employ the benchmark dose (BMD) method in toxicological risk assessment, it is critical to understand how the BMD lower bound for reference dose calculation is selected following statistical fitting procedures of multiple mathematical models. The purpose of this study was to compare the performances of various combinations of model exclusion and selection criteria for quantal response data.

Methods: Simulation-based evaluation of model exclusion and selection processes was conducted by comparing validity, reliability, and other model performance parameters. Three different empirical datasets for different chemical substances were analyzed for the assessment, each having different characteristics of the dose-response pattern (i.e. datasets with rich information in high or low response rates, or approximately linear dose-response patterns).

Results: The best performing criteria of model exclusion and selection were different across the different datasets. Model averaging over the three models with the lowest three AIC (Akaike information criteria) values (MA-3) did not produce the worst performance, and MA-3 without model exclusion produced the best results among the model averaging. Model exclusion including the use of the Kolmogorov-Smirnov test in advance of model selection did not necessarily improve the validity and reliability of the models.

Conclusions: If a uniform methodological suggestion for the guideline is required to choose the best performing model for exclusion and selection, our results indicate that using MA-3 is the recommended option whenever applicable.

Keywords: benchmark dose, model averaging, simulation

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