Rapid structural determination of persistent organic pollutants of agrochemical degradation products using the crystalline sponge method: Versatility of new-generation X-ray structural analysis on the submicrogram scale

For future production and use of agrochemicals based on green, sustainable chemistry, there are urgent demands for identifying agrochemical degradation metabolites occurring in soil, water, the atmospheric environment and crops, and for assessing their toxicities in view of food safety. In the determination of the chemical structures of such unidentified metabolites, a common approach is to synthesize these as reference standards based on the structures estimated from chemical information obtained from mass spectrometric analysis by precision mass spectrometric analysis using Liquid Chromatography / High-Resolution Mass Spectrometry (LC/HRMS), GC/HRMS and nuclear magnetic resonance (NMR) spectral analysis, and to identify unknown metabolites by comparing with the chemical identities of the reference standards. However, agrochemical degradation metabolites are usually obtained in only very small amounts, and a multitude of structures can occur in the environment and in crops. Therefore, synthesizing all such potential metabolites and similar candidate compounds is not a realistic endeavor due to the extreme time and economic efforts involved. This article thus describes a number of examples of successful analyses for the quick structural determination of extremely small amounts of organochlorine agrochemical degradation metabolites using the crystalline sponge method, a new method of X-ray-based structural determination.

What is the crystalline sponge method?

Single-crystal X-ray structural analysis (SCX) is an analytical technique in which an X-ray beam is applied to a crystal of a sample molecule to determine its molecular structure from the resulting X-ray diffraction pattern. The primary feature of SCX is the ability to directly determine the three-dimensional structure of the molecule that forms the crystal, and examine it as if the molecule was at hand. In this regard, SCX is distinct from NMR, MS, and other spectral analyses that necessitate spectral chart assignment for a puzzle-like assembly to hypothesize the original molecular structure. As such, SCX has long been established as the most reliable method of determining the molecular structures of a range of substances, from small molecules to proteins. As described above, SCX may be referred to as the “king of molecular structural analysis”. However, in reality, it is less popular than common-use spectral analysis.

The major drawback of SCX is the necessity of preparing a good-quality crystal of the target molecular species. Whether a molecule crystallizes successfully depends on its physicochemical properties and the experimental operator’s “good fortune.” For example, for one particular compound, only one particle of a crystal sample was obtained after many years of crystallization experiments under tens of thousands of conditions. Although this is an extreme case, crystallization research usually requires a relatively large sample amount, i.e., several milligrams. If such difficult issues associated with crystal preparation and sample volume requirement are resolved, the applicability of SCX would expand dramatically.

The crystalline sponge method is a groundbreaking method for preparing SCX samples, published by Professor Makoto Fujita and colleagues at the University of Tokyo in 2013. With this technique, analyte molecules (guest) are enclosed in a crystalline molecular container (host) known as a crystalline sponge to prepare a host–guest complex (Figure 1). The enclosed guest molecules are arranged in a regular pattern according to the periodicity of the host crystal. Therefore, the complex as a whole can be deemed as a single crystal sample. Structurally analyzing this crystal using SCX enables determination of the structures of the molecules enclosed in the crystalline sponge in exactly the same manner as with ordinary SCX.

The crystalline sponge method offers solutions to the two aforementioned issues concerning SCX. Because the host–guest complex is prepared using a highly crystalline host as the container, a crystal sample that is suitable for SCX can be prepared without being dependent on the crystallinity of the analytical sample itself. This allows compounds whose structures have traditionally been impossible to determine by SCX, such as liquids and gases, to be subjects of analysis as well. In addition, because an inclusion crystal is prepared using only one particle of crystalline sponge measuring about 1 cubic μm, a volume of analytical sample that fills the inside space is sufficient. As a result, the crystalline sponge method enables molecular structural analysis on the submicrometer scale, thus scaling-down experiments by more than one thousand-fold compared to the conventional crystallization-based method, for accurate structural determination in still smaller sample amounts compared to using NMR, etc.
Application to analysis of assumed persistent organic pollutants (POPs) agrochemical degradation metabolites

In this study, we determined the structures of selected degradation products from farmland-contaminating POPs agrochemicals, i.e. assumed hexachlorobenzene (HCB) degradation metabolites generated by aerobic, dechlorinating microbes (pentachlorophenol [PCP], 2,3,4,5-tetrachlorophenol [TCP], 2,3,5,6-TCP) in small sample amounts using the crystalline sponge method. Because these compounds are similar to each other in molecular structure and have few $^1$H nuclei, their structures are difficult to determine only using NMR and MS (Figure 2A).

First, PCP was investigated. Preparation of an inclusion crystal was implemented using reported methods. A 1 µg solution of PCP in cyclohexane was added to one particle of crystalline sponge and the solution was concentrated over several days to prepare an inclusion crystal. When the resulting inclusion crystal was structurally analyzed using SCX, a structure was revealed showing PCP molecules trapped in the pores of the crystalline sponge (Figure 2B). Selective display of the guest molecule alone clearly showed the PCP structure. Actually, the information obtained using SCX does not represent the molecular structure as it is, but instead indicates the shape of the electron density distribution in the crystal; therefore, it is necessary to assign atoms to respective electron density peaks. In this study, we were able to clearly distinguish between the substituent oxygen atoms and chlorine atoms in the PCP based on the difference in the magnitude of electron density (roughly proportional to atomic number). We succeeded in directly determining the structure of PCP using an extremely small sample amount totaling 1 µg.

Next, two types of TCP were investigated. A 5 µg solution of 2,3,4,5-TCP or 2,3,5,6-TCP in cyclohexane was added to one particle of each crystalline sponge to prepare inclusion crystals, which were then structurally analyzed by SCX. As with PCP, molecular structures of the TCPs were obtained (Figure 2C, D). Notably, the chlorine atom substitution sites in the two isomers were clearly able to be distinguished based on the electron density map. Because the two isomers have identical elemental composition and few $^1$H nuclei, they are difficult to distinguish by NMR or MS; however, their structural identification was enabled by directly determining the spatial arrangements of the atoms by SCX. As suggested by this case, degradation products resulting from the same substrate often have a similar structure, so SCX, which was able to clearly distinguish between them, represents a powerful tool for degradation product analysis.

In this article, use of the crystalline sponge method allowed for structural determination of assumed POPs agrochemical degradation metabolites using extremely small sample amounts. As no other approach is able to produce accurate structural determinations with just micrograms of unidentified sample purified by preparative HPLC, based on degradation/metabolism experiments on a laboratory scale, SCX thus offers the potential of dramatically changing the conventional modality of degradation metabolite analysis. This achievement will lead to the development of new agrochemicals with higher environmental and crop safety, and could allow the elucidation of detailed information on the fate (degradation metabolism pathways) of residual organic contaminants, such as existing agrochemicals and POPs, which have not been clarified thus far.
References


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