Chemical Identification and Confirmation of Contact Allergens

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Identification of the etiological chemical agent(s) associated with a case(s) of allergic contact dermatitis (ACD) is important for both patient management and public health surveillance. Traditional patch testing can identify chemical allergens to which the patient is allergic. Confirmation of allergen presence in the causative ACD-associated material is presently dependent on labeling information, which may not list the allergenic chemical on the product label or safety data sheet. Dermatologists have expressed concern over the lack of laboratory support for chemical allergen identification and possibly quantification from patients' ACD-associated products. The aim of this review was to provide the clinician a primer to better understand the analytical chemistry of contact allergen confirmation and unknown identification, including types of analyses, required instrumentation, identification levels of confidence decision tree, limitations, and costs.

New chemicals are continuously introduced into the market. Several animal-based screening methods (ie, guinea pig maximization test and the murine local lymph node assay) have been used to identify chemicals with contact allergenic potential; however, patients are continually diagnosed with allergic contact dermatitis (ACD) to previously unrecognized chemical allergens. Between 2008 and 2015, 172 new contact allergens were identified through patient patch testing (119 of these were associated with ACD) and reported in *Contact Dermatitis* and *Dermatitis*.[1] The actual number of new contact allergens is likely much higher because of incomplete product labeling, new allergens identified by patch testing but not reported in the literature, and those associated with ACD for which the specific chemical goes unidentified. In addition, contact allergic reactions may be identified only as a positive reaction to a personal or workplace material but cannot be explained by known allergens, and patch testing to personal/workplace materials is not always performed. For example, there have recently been several reports of the presence of undeclared formaldehyde and methylisothiazolinone in multiple cosmetics and other products.[2–5]

Multiple pop cultural television shows and movies portray situations where an investigator or technician injects a sample into an instrument and within minutes the instrument reports the chemical composition with absolute certainty. This has created an expectation among the general public/patients that the specific agent(s) causing their ACD can be quickly and easily identified. Unfortunately, chemical identification is rarely simple and is a topic that should be included in patient counseling.

A positive patch test demonstrates that the patient has been exposed to and has developed sensitivity to that particular allergen, but attributing ACD to that allergen is much more difficult. Clinical relevance of a positive reaction requires careful examination of current exposures. The patient's ACD may be due to multiple allergens and possibly an allergen(s) that is unrecognized. The patch test–positive allergen may also not be present in the associated materials. For example, a patient may present with ACD from use of a rubber product and have a positive mercaptobenzothiazole patch test, but mercaptobenzothiazole may not be found in that product upon chemical analysis.[6]

There are 2 main types of investigations related to allergen identification. The first is verifying the clinical relevance of an allergen identified by patch testing by confirming the presence or (relative) absence of that chemical allergen in the ACD etiological product (eg, analysis for diphenylguanidine in a glove from a patient found to have a positive patch test reaction to diphenylguanidine). The second is the challenging and often more costly endeavor to identify an unrecognized, potentially new allergen from a product that has triggered ACD in a patient. The additional step in either type of

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The authors have no funding or conflicts of interest to declare.

This study involved the use of resources and facilities at the Minneapolis Veterans Affairs Medical Center as well as the National Institute for Occupational Safety and Health of the Centers for Disease Control and Prevention. The contents do not represent the views of the US Department of Veterans Affairs or the US Government. The findings and conclusions of this report are those of the authors and do not necessarily represent the official position of the National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention. Mention of any company or product does not constitute endorsement by the National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention.

DOI: 10.1097/DER.0000000000000498

investigation of allergen quantification presents additional challenges and cost. Allergen quantification, although important for product screening for allergenic potential, is, in general, not needed for assessing the causative agent in an ACD-associated material and is not addressed in this study.

Chemical analyses may be essential from a public health aspect in identifying the etiological allergen involved in an ACD epidemic. This is particularly true when the contact allergen is tangentially related to the ACD-causing product content. The classic example, such as an outbreak, is dimethyl fumarate (DMF)–mediated furniture-related severe ACD as reviewed by Lammintausta et al.[7] The first cases involving Chinese-made recliner chairs were reported in Finland in 2006. Although the manufacturer denied addition of chemicals to the furniture materials, new cases of furniture dermatitis were identified in the United Kingdom. Dimethyl fumarate, a volatile solid fungicidal fumigant used in sachets to prevent mold overgrowth during transportation, was identified by traditional patch tests and by patch testing affected patients with thin-layer chromatography strips and then analyzing the segment of the strip causing a positive patch test reaction (chromatographic patch testing).[7,8] Since that initial outbreak, DMF ACD has been reported from clothing and wallets.[9–14]

Because of the utility of chemical laboratory–based studies in identifying undeclared allergens for specific patients as well as for public health in ACD epidemics, a basic understanding of such processes is valuable. The purpose of this study is to provide a general primer outlining methodologies and limitations for identification of a contact allergen(s) from materials associated with ACD cases.

CHROMATOGRAPHIC PATCH TESTING: IDENTIFICATION OF AN UNKNOWN ALLERGEN IN ACD-CAUSING MATERIAL

The Environmental Working Group and "a coalition of public interest and environmental health organizations" conducted a survey in 2004 of more than 2300 people and reported "the adult uses 9 personal care products each day, with 126 unique chemical ingredients" (*Exposures Add Up—Survey Results.* Environmental Working Group's Skin Deep Cosmetic Database [January–May 2004][15]; https://www.ewg.org/skindeep/2004/06/15/exposures-add-up-survey-results/). This presents a tremendous challenge in identifying the specific chemical agent(s) that elicits a patient's ACD.

Chromatographic patch tests have been developed to separate individual components from a product extract onto a platform amendable for use in patients' ACD patch testing. There are multiple chromatographic chemical separation techniques used with patch testing that have been reported in the literature. Almost all chromatographic methods are based on partitioning of the analytes between a stationary phase and a mobile phase (gas or liquid). Separations are achieved based on the relative affinities of the analytes for the chromatographic stationary and mobile phases (usually based on phase and analyte polarities with more polar solid phased materials having greater affinities for more polar analytes, ie, the "like dissolves like" rule).

The earliest report we found of a chromatographic patch test was that of Pirila and Rouhunkoski.[16] They separated bacitracin (a polypeptide) from its breakdown products using paper electrophoresis, which is a technique where a chemical mixture is applied to absorbent paper and placed in a buffer and a charge is applied across the system. The chemicals migrate across the paper according to their charge (+/–) density/strength. Using this technique, they were able to demonstrate that allergy was due to the parent compound (bacitracin) and not the breakdown products by patch testing the electrophoresed paper on the patient. Electrophoretic separation is more often used for peptides and proteins. Ten years later, Mijnssen[17] used a paper chromatographic technique to separate tulip chemical components based on their relative affinity between the paper and chromatographic solvent. The chromatographic paper with the individual tulip components separated across the paper was then used for patch testing tulip-allergic patients to identify the specific tulip allergenic chemicals.

Silica Gel Thin-Layer Chromatographic Technique

Bruze et al[18] reported the use of a thin-layer chromatographic (TLC) patch test, and Bruze and colleagues' laboratory has identified a number of allergens, including DMF, using TLC patch tests.[7,19,20] The silica gel technique TLC involves pipetting the chemical mixture onto a plate coated with an absorbent gel/film (called the stationary phase). Multiple solid-phase materials are available for achieving optimal chemical chromatographic separations. Silica gel normal-phase material (that absorbs more polar chemicals better and thus polar chemicals migrate slower up the plate) or reverse-phase TLC solid phases (absorbs nonpolar chemicals better and these move slower up the plate) are most commonly used to separate chemical mixtures. Thin-layer chromatography has been used in chemical separation science for many years, but recent advances allowing for flexible plastic TLC supports allow its use in patch testing. The TLC used by Bruze and colleagues' laboratory separated chemicals on a silica gel (normal-phase material). For this technique, the TLC plate is placed into a chamber with a solvent (mobile phase), and the chemicals migrate up the plate at different rates based on their polarity as the solvent moves up the plate. The silica gel TLC technique is very similar to paper chromatography where the chemical is spotted onto an absorbent paper, but in general, it is faster and provides better chemical spot resolution than paper chromatography. The plastic support also can be fluorescent, allowing easy chemical spot visualization by the blockage of the backing plate fluorescence by the chemical spot. The chemical from the spot corresponding to the positive patch test can be easily recovered for further chemical identification testing as described hereinafter (mass spectrometry [MS]).

There are several limitations to TLC including the following: potential false negatives (especially for less potent allergens due to the limited TLC sample loading causing insufficient allergen quantity

on the TLC strip to elicit ACD), incompatibility of some chemicals with TLC, and multiple chemicals in a single visualized spot. Several extracts using different solvents may be needed to ensure that the allergen is in the extract applied to the TLC plate, and different mobile phases may need to be tested to obtain the optimal chemical separation on the plate. Silica gel is very polar and not compatible with high water content chromatographic solvents (mobile phases). Conducting sample extraction and TLC procedures requires the use of a chemical safety cabinet/fume hood to protect the technician, and solvent disposal may also be an issue in setting up a TLC patch test clinic. For example, a TLC mobile phase solution containing chloroform and acetonitrile was used to separate dyes for patch testing[20,21]; both of these chemicals are potential occupational hazards. Reverse-phase TLC, which has greater affinity for nonpolar analytes, may be a safer option, occupationally, because alcohol-water mobile phases are commonly used, although compatibility of reverse-phase material, such as the nonpolar C-18 bound silica gel, would need to be assessed as a patch test media. Beyond chemical separation/isolation, the data obtained from TLC are very limited. Comparison of the distance a known chemical allergen standard migrates up the TLC plate to that of the allergen from the extract may be sufficient to confirm the identity of that suspected allergen for case management; however, in the absence of identical migration distances up the TLC plate, additional chemical analytical assessment is required.

Summary of TLC

The basic steps in the TLC patch test process are depicted in Figure 1. The basic steps are as follows. Initially, the material/product that contains the contact allergen is extracted using the appropriate solvent. The extraction may be concentrated if needed and then applied to a TLC plate/strip. The plate is then placed in a chamber with the appropriate mobile phase to separate the mixture into individual chemical components. The developed plate is allowed to dry to remove the mobile phase and taped to the subject's skin. The TLC strip is removed after 2 days and read as in standard patch testing (48 hours and 72–120 hours). A second TLC test strip developed under identical chromatographic conditions is marked at the spots corresponding to the subject's positive allergic reaction(s). These allergen-containing spots are scraped from the TLC strip, extracted, and used for subsequent chemical analyses.

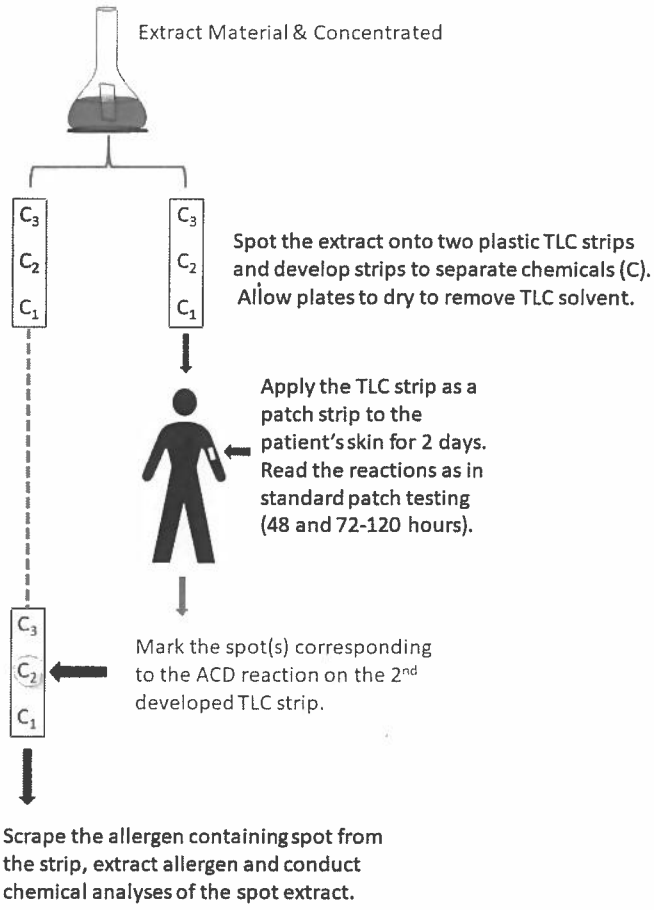

Figure 1. Thin-layer chromatographic patch test process flow diagram. The TLC patch test can be used to separate a complex chemical mixture from a product extract into individual chemicals on a chromatographic strip compatible for use in contact allergen patch testing. The essential steps are as follows: (*a*) extract and concentrate potential chemical allergens from the ACD causative material; (*b*) spot the extract onto TLC strips and develop the strips and dry to remove TLC solvents; (*c*) patch the developed TLC strip onto the patient/subject; (*d*) mark the specific spot(s) on a second developed TLC strip that corresponds to positive ACD reactions; and (*e*) recover the ACD-associated spots from the TLC plate and extract for subsequent chemical analyses.

CONFIRMATION TESTING: IDENTIFICATION OF A KNOWN ALLERGEN IN A MATERIAL

Spot Tests

Figure 2 outlines the pathways and decision processes in specific allergen content confirmation in an ACD causative material. The fastest, most economic analysis to confirm the presence of a suspect allergen in a material is by using a spot test. However, there are only a few chemical spot tests commercially available, and these are usually marketed only for analyses in water. We have tested several commercially available formaldehyde spot tests for use with cosmetic products and find that they have some utility in the clinical setting.[2] Spot tests are also commercially available for some metals, such as nickel, cobalt, and chromium, and these have been used in patch test clinics.[22–24] Additional information concerning testing for metals is provided in a separate section hereinafter. There is also a spot kit for identification of isothiazolinones (methylisothiazolinone/methylchloroisothiazolinone) in water; however, this has not been validated for use to detect these chemicals from consumer and nonconsumer commercial products. Commercially available spot tests are relatively inexpensive, fast, easy to run, and amenable to a clinical setting. They can be performed in a semiquantitative to quantitative manner. Potential interferences can arise from similar chemical classes, discoloration of the test strip, and chemical interference with formation of the colorimetric reaction product

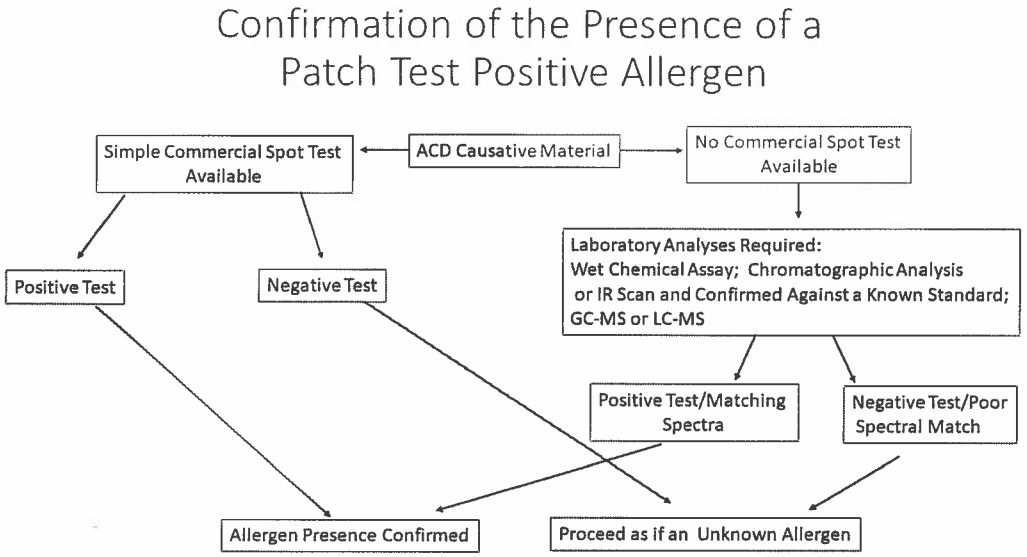

Figure 2. Confirmation of the presence of a patch test–positive allergen. The flow diagram is of possible steps for confirming the presence of a patch test–positive allergen in the patient's ACD-associated material/product.

or from color/dyes from the ACD-associated materials. Because most spot tests are designed for water assessment, chemical extraction from the ACD-associated material may be needed along with centrifugation or filtration of the extracts to remove insoluble components that interfere with reading the spot test color change.

Laboratory Analytic Chemistry Methods

Most chemical analyses require laboratory-based analyses. The typical dermatology clinic does not have the appropriate chemical safety and chemical waste disposal procedures required for analytical chemistry laboratories. For example, the ASTM International test method D7558 is a colorimetric/spectrophotometric assay for the measurement of (allergenic) accelerators from nitrile and latex gloves and uses acetonitrile as the extraction/assay solvent and cobalt to detect zinc dithiocarbamates and thiurams. Mercaptobenzothiazole can also be detected using a spectrophotometer by this assay. Although the assay is relatively simple, both acetonitrile- and cobalt-containing wastes are generated. In general, any chemical laboratory assay will generate potentially hazardous chemical waste for which disposal is highly regulated by the Environmental Protection Agency.

Analytical chemistry techniques to confirm the presence of a suspected allergen can range from relatively simple, inexpensive colorimetric assays to assays requiring the use of expensive, complex analytical equipment. As stated previously, identical TLC migration with the corresponding chemical analytical standard can provide a modicum of confirmation. Higher-resolution (with respect to separating mixtures into individual chemicals) chromatographic systems, such as high-performance liquid chromatographs (HPLCs, the mobile phase is a liquid) or gas chromatographs (GCs, the mobile phase is a gas) coupled to simple detectors, such as UV/VIS/diode array spectrophotometric or flame ionization detector (FID), respectively, very common and fairly nonspecific detectors, are usually adequate to confidently confirm the identity of a suspect allergen against a known chemical analytical standard. The diode array detector can provide the UV/VIS absorbance spectra of each chemical because it elutes from the HPLC, and the retention time and spectra can be compared with those of the suspected allergen. Because organic chemicals elute from a GC column, they are passed through a flame, and ions are generated that can be detected by the FID. Essentially, all organic chemicals will create ions when passed through the flame in the FID, and thus, chemically identified confirmation is based on comparison of GC-column retention of the allergen standard to the patient's ACD material-associated chemical.

Mass Spectrometry

In the absence of an analytical standard of the suspect allergen, HPLC-MS or GC–electron impact (EI)–MS is necessary. Chemical mass can be ascertained by HPLC-MS and tentative identification made by comparing the fragmentation pattern from GC-MS analysis against that from a standard library (eg, NIST) or EI-MS spectra from the literature. Additional information can be obtained from tandem MS systems where the chromatographic column effluent goes into 1 MS where it is ionized and selected ions are then directed to a second MS where they are fragmented into multiple product ions. The chemical mass can be determined from the first MS, and the product ions from the second MS can be evaluated for consistency with that expected from a proposed chemical structure. Use of a tandem MS system does increase the analysis cost and is usually not necessary to confirm the chemical identity of a suspect allergen.

IDENTIFYING AND ANALYZING UNKNOWN ALLERGENS FROM A PRODUCT OR DEVICE

As summarized in Figure 3 and previously, the processes involved in identifying an unknown chemical contact allergen usually involve a

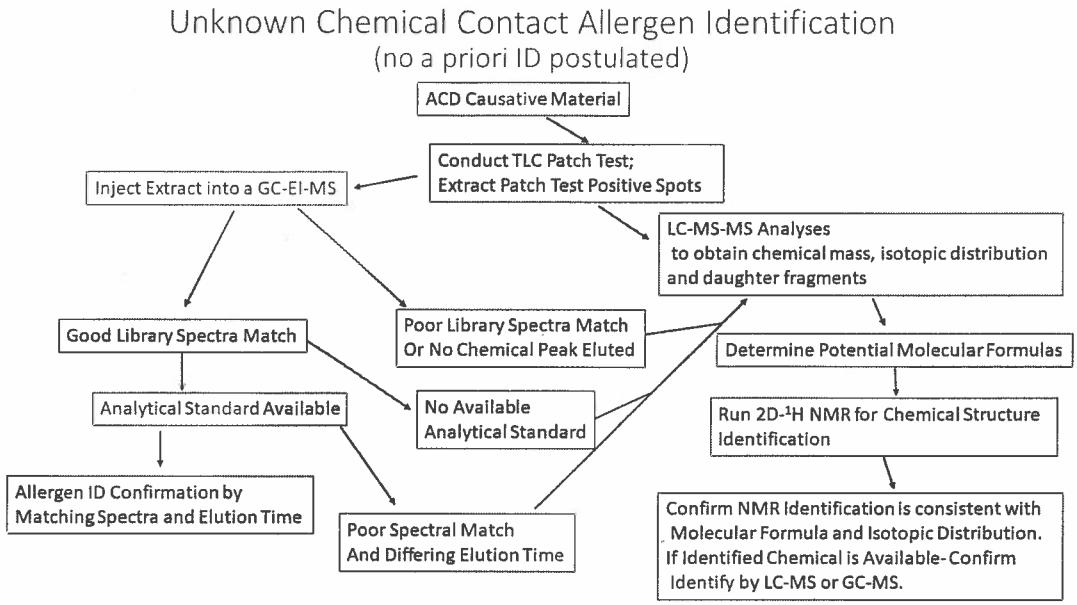

Figure 3. Unknown chemical contact allergen identification (no a priori ID postulated). The flow diagram outlines a protocol for elucidating the chemical structure of an unknown contact allergen. In general, the time, cost, and analytical chemical expertise required increase from the top, left to the bottom, and right of the diagram.

chromatographic patch test as a critical first step. The TLC spot can be easily scraped from the plate and the chemical(s) extracted from the TLC stationary phase (silica gel) material. Various levels of confidence in chemical structural identification are obtained depending on the supporting analyses as suggested by Schymanski et al.[25] The most commonly used chemical identification technique, as mentioned previously, is to inject a portion of the extract onto a GC-EI-MS and compare the MS spectra obtained against a library. The NIST 17 library contains GC-EI-MS and MS/MS spectra along with GC data including retention indices. This library contains 306,622 spectra from 267,376 compounds, and the MS/MS library contains spectra from other MS techniques. The Wiley EI-MS library contains more than 775,500 spectra for 599,700 compounds. The purchase price of the combined libraries is approximately US $10,000. These libraries' software provides match scores of library spectra to the spectra of interest. Even a high probability match alone should not be considered as a confirmed chemical structure identification. It has been noted that for chemicals with spectra in the library, in 19% of the cases, the library search algorithms did not list the correct chemical as the best match, although the correct chemical was among the top 10 spectra matches in 98% of the cases.[26] The library may provide a GC column retention time (GC method dependent) that can be compared with that found for the allergen, but comparison with an authentic reference standard run in parallel to the allergen to confirm identical retention time and spectra is optimal for a high-level confident identification. As Lefty Gomez (NY Yankees Pitcher, 1930s) stated, "It's better to be lucky than good," and this applies to determining the chemical identity from a spot on a TLC plate. The previously mentioned scenario with a good GC-MS library spectra match confirmed against a reference material is the "lucky" scenario. A decision not to pursue the acquisition of additional chemical information in the absence of a reference standard may be made if the tentative chemical identification is reasonably expected from or associated with the ACD causative material. In the absence of a reference standard, additional analyses can provide information, such as exact mass/molecular formula, MS/MS spectra, and type of chemical bonds (infrared or nuclear magnetic resonance [NMR] analyses) to increase confidence in the chemical identification, but such additional analyses may be cost prohibitive.

Not all chemicals are amenable to GC-MS analyses. The GC-MS injector is heated to a high temperature (usually >200°C) to volatilize the chemical. Many contact allergens are not sufficiently volatile or may decompose at GC injector temperatures. A chemical allergen can often be chemically derivitized to a more volatile/stable form that is compatible with GC-MS analyses, but this "shotgun" approach of applying various derivatizing reagents for a completely unknown allergen presents a scenario with a low probability of success. In addition, the chemical allergen spectra may not be found in an MS library. In the absence of "luck," additional chemical analyses for chemical identification are required as described in the following.

Allergen nominal mass can be obtainable from a number of (soft) ionization techniques that are used in liquid and/or gas mass spectrometry. Exact mass may be obtained from high-resolution mass spectrometers. A calculator is available online that generates a list of possible molecular formula from an accurate mass (http://www.chemcalc.org/mf_finder/mfFinder_em_new[27]). This, again, does not provide chemical structural information, but exact mass, along with a chemical's isotopic distribution and MS-MS data, can

provide tentative chemical candidates or help confirm a GC-EI-MS spectral assignment in the absence of a reference standard.

Computational MS

Although mass spectral fragmentation libraries continue to expand, structural elucidation of an unknown allergen remains very challenging and potentially very costly. The field of metabolomics (identification and quantification of chemical/drug low-molecular-weight metabolites) has spurred the development of computational methods for the identification of such metabolites from MS fragmentation trees and using "machine learning" to predict the molecular structure[28–30] At present, such programs are continuing to improve and in the future may become a viable tool for unknown contact allergen chemical identification.

Nuclear Magnetic Resonance Spectroscopy: Identifying Chemical Structure

Nuclear magnetic resonance spectroscopy is commonly used to obtain chemical structural information. Nuclear magnetic resonance spectroscopy can be used to confirm a chemical structure or to elucidate the structure of an unknown chemical. There are multiple NMR experiments/types and spectra that can be obtained. The simplest is a 1-dimensional proton spectrum (^{1}H-NMR). In this spectrum, the chemical shifts are measured in a magnetic field, and essentially the fewer electrons associated with a proton, the higher the chemical shift of that proton. One-dimensional NMR provides information related to the functional group composition of the chemical, but 2-dimensional NMR experiments are required to connect these functional groups for structural identification. Correlation spectroscopy and total correlation spectroscopy are common 2-dimensional techniques used for structural elucidation. For a more complete overview and primer of the use of NMR, see the study by Simpson et al.[31] Nuclear magnetic resonance spectroscopy requires a pure chemical at much greater quantities than needed for MS techniques. The quantity of an unknown from a TLC patch test may be inadequate with respect to quantity and possibly purity required for NMR. Preparative TLC or additional preparative liquid chromatographic procedures may be required to obtain sufficient amounts and purity for NMR analyses.

INORGANIC (METALS) IDENTIFICATION

Simple wet chemical test and spot test for various metals can be performed on product extracts relatively inexpensively. For example, we have quantified nickel(II) from patch test reagents by reacting it with ammonia hydroxide to form a hexamine complex with a bright blue color that can be quantified spectrophotometrically.[32] Another nickel spot test that is commercially available is the dimethylglyoxime test kit, which is marketed for presence of nickel off a cotton swab from a metallic item. The dimethylglyoxime spot test has been reported to have good specificity (97.5%), but only "modest" sensitivity (59.3%).[24] Commercial spot test kits are also available for cobalt from swabs of metallic items. Hexavalent chromium reacts with diphenylcarbazide in an acid solution to form a red-violet product and has been used as a spot test for Cr(VI) release from leather and metals.[22] Elemental metal quantification can also be performed after digestion by inductively coupled plasma emission–mass spectrometry or inductively coupled plasma emission–atomic emission spectrometry.

COST AND FEASIBILITY

The cost of identifying or confirming the presence of a specific chemical allergen can range from a few dollars when using a spot test (test strip) to potentially thousands of dollars because specialty testing requires expensive, complex instrumentation and greater levels of chemistry expertise to interpret the results. Simple technical-grade chemicals may cost less than US $100, but less common chemical standards are very expensive. For example, a single urushiol congener standard (ie, a single component of the complex 3-alk(en)catechols that make up the more allergenic components of poison ivy oil) cost approximately US $900/10 μL in 2019. The goal of chemical identification can be roughly divided into that for clinical patient care or for public health purposes. At present, a patient's medical insurance will not cover the cost of allergen chemical identification, and the clinician needs to weigh the cost/benefit of identifying or confirming the specific chemical allergen(s) versus only identifying the ACD-associated material and conducting the appropriate standard patch testing with respect to counseling the patient on allergen avoidance. While both academic and commercial laboratories exist that contain the instrumentation and expertise to analyze and identify the chemical composition of an unknown substance, and there are commercial laboratories that specialize in product reverse engineering/deformulation, the cost burden is usually too great to go beyond confirmation of a suspected allergen using a simple, inexpensive spot test.

SUMMARY

Contact allergen chemical identification is important not only for individual patients but also for public health. The goals of public health are to prevent disease and to promote health in the general population, or a subsector thereof. Although the dermatologist may use the specific chemical identified to help counsel the patient in allergen avoidance, this identification may be relevant as a sentinel event with public health relevance in the prevention of an ACD outbreak. An assessment would need to be made of the potential disease burden and overall impact of identifying the specific agent inducing ACD. As described earlier, the classic example of an ACD "epidemic" is one that was initially identified from patients reacting to furniture imported from China with the etiological chemical allergen identified as the antifungal fumigant, DMF, using both TLC patch testing and GC-MS chemical analyses. This review summarizes the key steps and techniques for allergen chemical identification and confirmation.

ACKNOWLEDGMENT

The authors thank Ms Kimberly Clough-Thomas for her assistance with graphics.

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