

An in vitro method for predicting sensitizing properties of inhaled chemicals

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WASS U, BELIN L. An in vitro method for predicting sensitizing properties of inhaled chemicals. *Scand J Work Environ Health* 1990;16:208–14. A method for monitoring chemical reactivity in aqueous solutions, at neutral pH and 37°C, was developed. The chemical was allowed to react with a lysine-containing peptide, and the reaction was monitored with high-performance liquid chromatography. Simple acids, bases, and solvents did not react with the peptide, whereas isocyanates, anhydrides, and chloramine-T, substances well known for their sensitizing and asthma inducing properties, did. Thus a positive test strongly suggested that the chemical had the potential to act as a hapten and cause sensitization when inhaled. Prepolymers of diphenylmethane diisocyanate were considerably more reactive than prepolymers of toluene diisocyanate or hexamethylene diisocyanate. Isocyanates blocked with caprolactam, butanone oxime, malonic acid diethylester, or isononyl phenol showed no reactivity. This result suggested a significantly reduced risk of respiratory reactions when such blocked isocyanates are handled at room temperature. One blocked isocyanate showed, however, considerable reactivity.

Key terms: allergy, asthma, blocked isocyanates, hapten, respiratory tract sensitization.

It is known that some chemicals with a low molecular weight can induce respiratory symptoms similar to an allergic type I reaction. The clinical features indicate that the symptoms may, at least in some cases, be caused by a true type I hypersensitivity reaction. Associations between allergy-like respiratory symptoms and specific immunoglobulin (Ig) E antibodies have been demonstrated for isocyanates (1–19), reactive textile dyes (20–24), acid anhydrides (25–29), platinum group metal salts (30, 31), and the disinfectant chloramine-T (p-toluenesulfonamide, n-chloro, sodium salt, trihydrate; CAS 127-65-1) (32, 33). Furthermore, traces of the sterilizing gas ethylene oxide in hemodialysis filters have been shown to sensitize patients undergoing treatment (34–48).

A substance that is able to bind to a protein or other macromolecule in vivo and act as a hapten can also be anticipated to cause disturbances in the respiratory tract through mechanisms other than immunologic ones. Such substances may, for instance, interfere with receptor proteins or enzymes. This phenomenon has been suggested, on the basis of in vitro studies, for the reactive isocyanates (49). Furthermore, a recent in vitro study suggested that the pharmacological effect of toluene diisocyanate (TDI) may result from an autonomic imbalance between cholinergic and B-adrenergic neural control (50). The known respiratory effects of isocyanates have been reviewed by Musk et al (51).

The hypothesis behind this present work was that there ought to be a correlation between the ability of

a substance to react with proteins and its potential to induce respiratory tract dysfunction. Furthermore, we assumed that a well-chosen peptide could replace the protein in an in vitro assay. The use of a peptide was considered advantageous, as we anticipated technical difficulties in demonstrating, and quantifying accurately, the reaction between a chemical with a low molecular weight and a protein. Thus the objective of this work was to develop a method of monitoring the reaction between a chemical and a suitable peptide.

The choice of the amino acid sequence of the peptide was based on previously published data. Lysine was our first choice, as in vitro studies had shown that, when added to proteins, isocyanates and anhydrides react with this amino acid (52, 53). Lysine has an epsilon amino group which can take part in the reaction and has also been shown to be the most likely residue for the covalent binding of chloramine-T to a protein, although tyrosine, methionine, cysteine, and histidine were also modified by the substance (54). Furthermore, lysine has been shown to be involved in the reaction between proteins and ethylene oxide (55).

The second amino acid chosen was tyrosine. This amino acid absorbs ultraviolet light and can thus be detected easily in high-performance liquid chromatography (HPLC). Tyrosine has also been shown to be involved in the reaction between chloramine-T and proteins (54). On the basis of these considerations, three peptides containing lysine-tyrosine were investigated.

After a suitable test procedure had been established, we estimated the reactivity of various chemicals and industrially used isocyanate products to see if the reactivity was related to known respiratory effects of the substances.

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Material and methods

Reagents

The peptides L-lysyl-L-tyrosyl-L-lysine · 2 formate (LTL) (molecular weight 530), L-lysyl-L-tyrosyl-L-glutamic acid (LTG) and L-lysyl-L-tyrosine · acetate (LT) were purchased from Bachem Feinchemalien AG, Bubendorf, Switzerland. Tetrahydrofuran (for chromatography) and molecular sieve 3 Å (2-mm beads) were obtained from E Merck, Darmstadt, Federal Republic of Germany. Acetonitrile (CH₃CN, HPLC grade), 1-octane sulfonic acid sodium salt (HPLC grade), and 1-pentane sulfonic acid sodium salt (HPLC grade) were obtained from Fisons, Loughborough, England. The other reagents used were of analytical grade. Test-tubes (labeled KIMAX, USA, length 10 cm, diameter 13 mm) with screw caps and teflon-faced rubber liners were purchased from Kebo Lab, Stockholm, Sweden.

Industrially used isocyanates were collected from a Swedish polyurethane factory or were obtained from Bayer AG, Leverkusen, Federal Republic of Germany. Three reactive textile dyes (from the "Remazol" series produced by the Hoechst company) were collected from a Swedish dye house. These dyes were Remazol Black B (color index reactive black 5), Remazol Brilliant Blue R Spec (color index reactive blue 19) and Remazol Brilliant Yellow 4GL (color index reactive yellow 160).

Acetonitrile and tetrahydrofuran, used to dilute the test samples, were dried for 24 h with 10 g of molecular sieve per 100 ml.

High-performance liquid chromatography

For reversed phase HPLC, Nova-Pak C18 Radial-PakTM cartridges (4 µm particles, column length 10 cm, inner diameter 8 mm) and µ-Bondapak C18 Guard-PakTM precolumn inserts were obtained from Waters Associates, Inc, Milford, Massachusetts, United States.

The mobile phase in the HPLC consisted of 58 % methanol and 42 % aqueous buffer (volume %). The buffer was prepared by dissolving 5 g of 1-octane sulfonic acid sodium salt, 1.5 g of 1-pentane sulfonic acid sodium salt, and 1 ml of triethylamine per liter of water. Before the buffer was used, its pH was adjusted to 3.0 by the addition of 10 volume % phosphoric acid.

The samples were chromatographed at a flow rate of 2 ml/min with ultraviolet detection at 280 nm. Ambient temperature and an injected volume of 50 µl were used. The reproducibility in HPLC was investigated by 12 consecutive injections of an 0.1 µmol/ml (53 µg/ml) solution of LTL. Overall variations in the test system were estimated by testing 10 industrially used isocyanate products, repeating the test after 2 d, and studying the performance of duplicate samples according to the method described by Barnett (56).

Pilot experiments

The reaction between isocyanates and peptides was studied as a model for all reactive chemicals. Various combinations of isocyanate and peptide concentrations were used, and several mobile phases were tested. The reaction between ethylene oxide, at a high concentration, and LTL was also studied.

Test procedure

LTL was dissolved in an 0.02 mol/l sodium phosphate buffer (a mixture of 0.02 mol/l sodium dihydrogen phosphate and 0.02 mol/l sodium hydrogen phosphate pH 7.0) to a final concentration of 0.1 µmol/ml. Aliquots of the solution were used the same day or stored frozen at -20°C.

The test sample was dissolved in water to a final concentration of 4.0 µmol/ml. Samples that were not soluble, or stable, in water were dissolved in acetonitrile instead.

To test a sample, 50 µl of the sample solution (4.0 µmol/ml) was added to 500 µl of the LTL solution (0.1 µmol/ml) in a test-tube at ambient temperature. The capped test-tube was then immediately placed in a 37°C water bath for 10 min and, before injection, in a 20°C (ambient temperature) water bath for 1 min.

During the HPLC, 50 µl of the mixture was injected, and the area for the LTL peak was measured. This peak area was compared with the area for the unreacted LTL peak, ie, the peak obtained when 50 µl of water (or acetonitrile) was mixed with 500 µl of the LTL solution. The percentage of LTL that had reacted with the test sample was calculated, divided by 10, and rounded off to the nearest integer. The resulting figure was named the "peptide reactivity index," and it ranged from 0, when no detectable reaction could be noted, to 10, when all LTL had reacted.

Industrially used isocyanates

Products based on hexamethylene diisocyanate (HDI), diphenylmethane diisocyanate (MDI), toluene diisocyanate (TDI), isophoron diisocyanate (IPDI), and naphthylene diisocyanate (NDI) were tested.

As the molecular weight of many industrially used isocyanates was not known (or could not be defined) and as mixtures were often used, a slight modification of the procedure that has already been presented was adopted for such samples. A hypothetical molecular weight of 500 was assumed, ie, 4 µmol/ml corresponded to 2 mg/ml.

Furthermore, some isocyanate products were difficult to dissolve directly in acetonitrile. These products were dissolved in tetrahydrofuran (20 mg/ml) as a first step and then diluted with acetonitrile to 2 mg/ml.

Thus 50 µl of the isocyanate solution (2 mg/ml in acetonitrile) was added to 500 µl of the LTL solution (0.1 µmol/ml) in a test-tube and treated as has already been described.

Results

Pilot experiments

Acetonitrile could be used as a solvent as it did not affect the peptides. All three peptides (LTL, LTG and LT) had the ability to react with the isocyanates MDI, TDI (a mixture of the 2,4- and 2,6-isomers) and HDI. LTL was, however, found to be the peptide with the longest retention time. Generally, LTL was the peptide that showed the best separation from the other components in the chromatograms. For this reason, LTL was chosen for the experiments that followed. The detection limit of LTL was approximately 0.001 $\mu\text{mol/ml}$.

The relative standard deviation [coefficient of variation (CV)] of the HPLC, investigated by 12 consecutive injections of an 0.1 $\mu\text{mol/ml}$ (53 $\mu\text{g/ml}$) solution of LTL, was 2 %. The standard deviation for the complete test procedure, estimated from tests of isocyanate products on two different occasions and the determination of the differences between duplicate samples, corresponded to approximately 4 % of the original

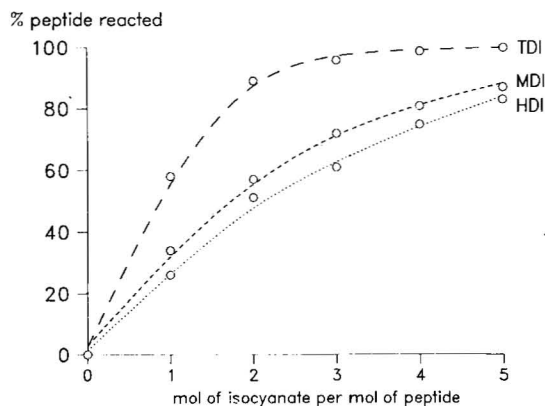


Figure 1. The percentage of the L-lysyl-L-tyrosyl-L-lysine (LTL) peptide that reacted for various isocyanate:LTL molar ratios. Diphenylmethane diisocyanate (MDI), toluene diisocyanate (TDI), and hexamethylene diisocyanate (HDI) were allowed to react with LTL (0.1 $\mu\text{mol/ml}$) at 37°C for 10 min.

LTL peak area. The retention time for LTL decreased linearly as the column temperature increased. The retention time at 50°C was approximately half that obtained at 20°C. Ambient temperature was chosen, but the experiment showed that temperature was a variable that could have been changed if the LTL peak had not been fully resolved in the chromatogram.

MDI, TDI, and HDI were allowed to react with LTL (0.1 $\mu\text{mol/ml}$) at various molar ratios (figure 1). The percentage of the peptide that reacted increased as the isocyanate:peptide ratio increased, and the differences between the three isocyanates were noted. The potential to react with LTL was highest for TDI, followed by MDI and HDI in that order. From the results illustrated in figure 1, a molar ratio of 4 between the test sample and the LTL peptide was chosen. At this ratio, TDI reached the 100 % level of reacted peptide. Furthermore, differences between the individual isocyanates were clearly noted, whereas a considerably higher ratio would eliminate this difference. A typical chromatogram from a test of HDI is shown in figure 2. As can be seen, HDI itself did not produce peaks that interfered with LTL. It will also be seen that only about 30 % of the original LTL peak remained after the reaction with HDI, which corresponded to a peptide reactivity index of 7. The reaction products of HDI and LTL were probably represented by additional peaks with retention times between approximately 2 and 4 min (figure 2, chromatogram D).

No detectable reaction was noted between ethylene oxide and LTL at a molar ratio of 4.0. A clear decrease in the LTL peak area was, however, observed when the ratio was increased to 450 and the reaction was monitored for 3 h. Under these conditions, an amount of LTL corresponding to approximately 15 % of the original amount was reacted per hour.

Chemicals

Several well-defined chemicals were allowed to react with LTL for 10 min at 37°C and at a molar ratio of 4. The same procedure was also used for three reactive textile dyes collected directly from a dye house.

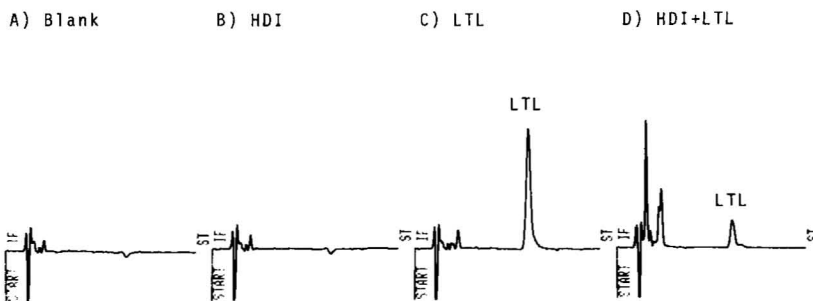


Figure 2. Chromatograms from a test of hexamethylene diisocyanate (HDI) according to a normal test procedure [0.1 $\mu\text{mol/l}$ L-lysyl-L-tyrosyl-L-lysine (LTL), HDI:LTL molar ratio 4]. The retention time for LTL was 8.5 min. [A] blank: acetonitrile added to buffer, [B] HDI: HDI in acetonitrile added to buffer, [C] LTL: acetonitrile added to buffer with LTL, [D] HDI + LTL: HDI in acetonitrile added to buffer with LTL]

Table 1. Results obtained for well-defined chemicals of analytical grade and for three reactive textile dyes collected directly from a dye house. In the test, 50 μ l of the sample solution (4.0 μ mol/ml) was added to 500 μ l of the L-lysyl-L-tyrosyl-L-lysine (LTL) solution (0.1 μ mol/ml), corresponding to a molar ratio of 4 between the sample and the LTL peptide. The solvent used for the tested chemical is indicated in the table.

Chemical substance	Solvent	Peptide reactivity index ^a
Acids/bases		
Ammonium hydroxide (NH ₃ aq)	Water	0
Formic acid	Water	0
Hydrochloric acid	Water	0
Perchloric acid	Water	0
Amines		
3-Dimethylamino-1-propylamine	Water	0
Ethanolamine	Water	0
Ethylenediamine	Water	0
Triethylamine	Water	0
Anhydrides		
Hexahydrophthalic anhydride	Acetonitrile	9
Maleic anhydride	Acetonitrile	10
Phthalic anhydride	Acetonitrile	10
Tetrachlorophthalic anhydride	Acetonitrile	7
Trimellitic anhydride	Acetonitrile	10
Dinitrobenzenes		
1-Chloro-2,4-dinitrobenzene	Acetonitrile	0
1-Fluoro-2,4-dinitrobenzene	Acetonitrile	0
Isocyanates		
Diphenylmethane diisocyanate (MDI)	Acetonitrile	8
Isophoron diisocyanate	Acetonitrile	5
Hexamethylene diisocyanate (HDI)	Acetonitrile	7
Phenyl isocyanate	Acetonitrile	7
Toluene diisocyanate (TDI)	Acetonitrile	10
Toluene-4-monoisocyanate	Acetonitrile	9
Miscellaneous		
Ammonium hexachloroplatinate (IV)	Water	0
Chloramine-T	Water	5
Ethyl methacrylate	Acetonitrile	0
Ethylene oxide	Water	0
Formaldehyde	Water	0
Hydrogen peroxide	Water	0
Isobutyl chloroformate	Acetonitrile	7
2-Mercaptoethanol	Water	0
2-Methylbenzoylchloride	Acetonitrile	0
N,N'-Methylenediacylamide	Water	0
Phenacyl bromide	Acetonitrile	0
Phenol	Water	0
Sodium persulfate	Water	0
Sodium sulfite	Water	0
Toluene-4-sulphonamide	Acetonitrile	0
Organic solvents		
Acetone	Water	0
Butanol (Butan-1-ol)	Acetonitrile	0
n-Butyl acetate	Acetonitrile	0
Dichloromethane	Acetonitrile	0
Diisopropyl ether	Acetonitrile	0
Propanol (Propan-2-ol)	Water	0
Reactive textile dyes		
Remazol Black B	Water	0
Remazol Brilliant Blue R Spec	Water	0
Remazol Brilliant Yellow 4GL	Water	0

^a See Test Procedure in the Material and Methods section for an explanation of the index.

The results, expressed as the peptide reactivity index, are shown in table 1.

The industrially used isocyanate products were tested at a concentration of 2 mg/ml, ie, a hypothetical molecular weight of 500 was assumed. The results, grouped according to the type of isocyanate product, are shown in table 2. The blocked isocyanates were dissolved in tetrahydrofuran (20 mg/ml) as a first step and then diluted with acetonitrile to 2 mg/ml. Monomeric isocyanates had been found previously to elicit the same peptide reactivity index regardless of whether

Table 2. Results obtained for industrially used isocyanate products. In the test, 50 μ l of the isocyanate solution (2 mg/ml in acetonitrile) was added to 500 μ l of the L-lysyl-L-tyrosyl-L-lysine (LTL) solution (0.1 μ mol/ml). The isocyanates are grouped according to the type of product. (N = the number of isocyanate products tested within each group, HDI = hexamethylene diisocyanate, IPDI = isophoron diisocyanate, MDI = diphenylmethane diisocyanate, NDI = naphthylene diisocyanate, TDI = toluene diisocyanate)

Type of isocyanate	Peptide reactivity index ^a	N	Comment
Mainly monomers			
MDI	7–10	7	-
TDI	10	1	80 % 2,4-TDI and 20 % 2,6-TDI
NDI	9	1	-
IPDI	9	1	-
Prepolymers			
HDI	1–3	5	-
TDI	1–3	4	-
MDI	5–10	4	-
Blocked isocyanates			
HDI	0	1	Blocked with butanone oxime
IPDI	0	1	Blocked with butanone oxime
MDI	0	1	Blocked with malonic acid ester
TDI	0	2	Blocked with caprolactam
TDI	0	1	Blocked with isononyl phenol
TDI	0	1	Blocked with aliphatic alcohol
TDI	3	1	Blocked with phenol
Hardeners for polyurethane lacquers			
HDI	2–3	2	-

^a See Test Procedure in the Material and Methods section for an explanation of the index.

they were directly dissolved in acetonitrile or tetrahydrofuran as a first step.

Discussion

Chemical reactivity is a relative concept. Industrially used chemicals may be reactive in organic solvents, at elevated pressures and temperatures, at extreme pH values, in the presence of catalysts and initiating substances, etc. The reactivity we wanted to estimate was that relevant to biological conditions, ie, aqueous solutions, neutral pH, and a temperature of 37°C.

Several reactive industrial chemicals have been found to exert sensitizing and toxic effects, presumably by interaction with mucosal surface proteins. An *in vitro* test system simulating this *in vivo* protein interaction was designed. We wanted to work with diluted solutions, since the concentration of inhaled chemicals is generally low. However, the analytical difficulties and the influence of trace impurities in solvents and buffers increase when low concentrations are to be analyzed. We found an LTL concentration of 0.1 μ mol/ml to be a suitable compromise.

For statistical reasons, the decrease in the LTL peak area for a single injection of a sample had to be at least approximately 5 % to be considered significant on the 99 % confidence level (normal distribution, one-tailed test). We wanted to report the results on a scale on which each increment represented a significant difference, and a scale from 0 to 10 was chosen.

Table 1 clearly shows that LTL was not affected by simple acids, bases, and solvents. Isocyanates, anhydrides, and chloramine-T, on the other hand, elicited peptide reactivity indexes in the range of 5 to 10.

It should be noted that not all substances known to sensitize the respiratory tract could be shown to react with the LTL peptide at a molar ratio of 4. For instance, the three reactive dyes tested did not. In this case the explanation may be that such dyes act as haptens without any covalent binding to a protein at neutral pH. Reactive dyes are normally activated by alkali before they become reactive and are able to bind covalently to textile fibers. Several reactive dyes have been shown to bind to human serum albumin and human IgG through adsorption (57-59), and we have previously suggested that covalent bonds may not always be necessary for the *in vivo* formation of dye-protein conjugates (24). Thus reactivity does not seem to be a prerequisite for hapten sensitization.

The reactivity of ethylene oxide seems to be too low to induce hypersensitivity reactions when inhaled. We found no specific IgE antibodies among 20 workers who worked in a plant producing ethylene oxide and were suspected of having work-related respiratory symptoms (Wass & Belin, unpublished data). However, hypersensitivity reactions to ethylene oxide have occurred among dialysis patients when the gas has had direct access to the blood in the dialysis filter. These circumstances might be reflected in the peptide test, in which no reaction was noted at a low ethylene oxide concentration (molar ratio 4, 10 min), but the substance revealed its reactivity clearly when the molar ratio was increased to 450 and the reaction was monitored for several hours.

One major application of this study concerns isocyanates, a group of reactive chemicals which has achieved ever-increasing technical importance in the last couple of decades. They are the starting materials for polymers called polyurethanes. Polyurethanes are used as flexible foam, rigid foam, electrical insulation material, lacquers, elastomers, glues, etc. The health hazards associated with isocyanates have been known for many years, and the use of these substances is regulated in Sweden and many other countries.

In spray painting, isocyanate-based coatings are used for automobiles, airplanes, railroad cars, furniture, etc. The necessary spray equipment is easier to produce and handle if a one-component paint is used (all ingredients are premixed in the paint) than if two-component systems (the isocyanate is mixed with the other ingredients during the spray operation) are used. The search for one-component systems that are stable enough to be stored for several months has led to products in which the reactive isocyanate (NCO) groups are blocked at ambient temperatures. Caprolactam, butanone oxime, and malonic acid diethylester have been used as blocking agents for isocyanates (60). Temperatures above 100°C are needed to release the blocking of the NCO

groups. Our data (table 2) indicate that isocyanates blocked with these three agents are inactive at 37°C. This finding suggests a significantly reduced risk of respiratory reactions when such blocked isocyanates are handled at room temperature, compared with prepolymers or monomers of isocyanates. From a theoretical point of view, an enzymatic degradation of blocked isocyanates *in vivo* to the corresponding free isocyanate (with NCO groups) may be claimed but, as far as we know, this hypothesis has not been supported by either *in vivo* or *in vitro* experiments.

The blocked isocyanate with phenol as the blocking agent, on the other hand, showed considerable reactivity. We tested two different batches of this product with the same result, peptide reactivity index 3; phenol itself did not react with LTL. Isocyanates blocked with phenol represent an old type of product that has been used mainly for wire coatings. It is interesting to note that the only report, as far as we know, of respiratory symptoms in regard to an occupational exposure to blocked isocyanates describes a man who sprayed with an adhesive containing a phenol-blocked TDI (61). Isononyl phenol, on the other hand, seemed to be an efficient blocking agent.

We found that prepolymers of MDI were considerably more reactive than prepolymers of TDI or HDI. This difference was obviously due to the high content of monomers in the MDI prepolymer, in which monomer concentrations of up to 45 % may exist.

Isobutyl chloroformate ($\text{ClCOOCH}_2\text{CH}(\text{CH}_3)_2$, CAS 543-27-1), a chemical used in analytical chemistry for the activation of carboxyl groups (62, 63), was found to have a peptide reactivity index of 7. This finding suggests a potential risk of respiratory symptoms if the substance is inhaled. We know of no such reports, but the substance is probably handled mostly by experienced chemists using fume hoods and other protective procedures.

We think that a positive test with the present peptide method strongly suggests that the tested chemical has, when inhaled, the potential to act as a hapten or to cause nonimmunologic disturbances (eg, interference with receptor proteins or enzymes). A negative test, on the other hand, does not exclude this possibility. The test result itself is not enough to judge a substance regarding its health hazards but may provide an important piece of information.

Several of our results could have been anticipated from what was known of the chemical properties of the substances. For instance, as phthalic anhydride was known to be a reactive substance, it was natural to expect that the same would apply to trimellitic anhydride. The peptide method can still be useful for substances with well-known chemical properties, however, because the method makes it possible to quantify the reactivity. Furthermore, the method can be applied to the testing of both complex mixtures and new chemicals with largely unknown properties.

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