

Stability of selected volatile contact allergens in different patch test chambers under different storage conditions

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Summary

Background. Patch test preparations of volatile substances may evaporate during storage, thereby giving rise to reduced patch test concentrations.

Objectives. To investigate the stability of selected acrylates/methacrylates and fragrance allergens in three different test chambers under different storage conditions.

Methods. Petrolatum samples of methyl methacrylate (MMA), 2-hydroxyethyl methacrylate (2-HEMA), 2-hydroxypropyl acrylate (2-HPA), cinnamal and eugenol in patch test concentrations were stored in three different test chambers (IQ chamberTM, IQ UltimateTM, and Van der Bend[®] transport container) at room temperature and in a refrigerator. The samples were analysed in triplicate with high-performance liquid chromatography.

Results. The decrease in concentration was substantial for all five allergens under both storage conditions in IQ chamberTM and IQ UltimateTM, with the exception of 2-HEMA during storage in the refrigerator. For these two chamber systems, the contact allergen concentration dropped below the stability limit in the following order: MMA, cinnamal, 2-HPA, eugenol, and 2-HEMA. In the Van der Bend[®] transport container, the contact allergens exhibited acceptable stability under both storage conditions, whereas MMA and 2-HPA required cool storage for maintenance of the limit.

Conclusion. The Van der Bend[®] transport container was the best device for storage of samples of volatile contact allergens.

Key words: acrylates; cinnamal; eugenol; GC-MS; HPLC; methacrylates; stability; volatile allergens.

Recent publications have demonstrated that selected acrylates/methacrylates and fragrance allergens disappear over time during storage in syringes, Finn

Chambers[®] and IQ chambersTM at room temperature and in the refrigerator (1, 2). The described loss of concentration of volatile allergens may give rise to false-negative patch test results in patients with suspected contact allergy, if filled test chambers are stored for even a few hours prior to the scheduled patch testing.

Furthermore, recent studies reported that the starting concentrations of isocyanates, disperse dyes and acrylates/methacrylates in fresh commercial patch test preparations were lower than stated by the producers (2–5). These findings are of great clinical importance, and are particularly relevant to the function of the Allergen Bank at the Department of Dermatology, Odense University Hospital (6). The Allergen Bank provides, on

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individual request, special contact allergens to dermatologists in practice. The contact allergens are shipped in test chambers by mail. Depending on the location of the dermatologist's practice, the shipment may take a few days to be received. It is therefore important to find a patch test chamber/container in which volatile contact allergens would be stable and consequently could be used for shipment. Additionally, we investigated the evaporation characteristics of cinnamal and methyl methacrylate (MMA), and the homogeneity of 2-hydroxyethyl methacrylate (2-HEMA) distribution in a fresh commercial patch test preparation. The selection of contact allergens for the present study was based on the expected volatility (higher versus lower vapour pressure) and previously published analytical data for the selected contact allergens.

Materials and Methods

Reagents

High-performance liquid chromatography (HPLC)-grade heptane, butanol and ethanol were obtained from Rathburn Chemicals (Walkerburn, UK). The selected patch test chambers/containers were IQ chamber™, IQ Ultimate™ (Chemotechnique Diagnostics, Vellinge, Sweden) and the Van der Bend® transport container (Van der Bend BV, Brielle, The Netherlands) (Fig. 1). The fresh commercial patch test preparations were purchased from Chemotechnique Diagnostics, and the analytical standards were obtained from Sigma-Aldrich (Steinheim, Germany). The solvents and test materials were filtered through a 0.45-µm organic nylon filter (Frisenette Aps,

Knebel, Denmark). The accurate weighing of test material was achieved by using analytical scales with a precision of 0.01 mg (XS 205 Dual Range; Mettler Toledo, Glostrup, Denmark).

HPLC apparatus and chromatographic conditions

An Agilent Technologies HPLC 1200 series comprising a G1315D Diode Array Detector, G1329A Automatic Liquid Sampler, G1316A Thermostatted Column Compartment, G1322A Vacuum Degasser and G1311A Quarternary Pump (Agilent Technologies, Santa Clara, CA, USA) was used. The detector was operated between 190 and 400 nm, and the contact allergens were detected at 215 nm. Processing and integration of the chromatographic data was performed with Agilent CHEMSTATION software (Revision B.04.01, Service Pack 1). Chromatography was carried out on a Luna 3 µ Silica (2) 100A column, that is, normal-phase chromatography (150 × 3.0 mm internal diameter; particle size 3.0 µm; Phenomenex, Torrance, CA, USA). A silica precolumn (4 × 3.0 mm; Phenomenex) was fitted, and served as a column protection. The column temperature was set at 25°C. The eluent composition was 99% heptane + 1% butanol (MMA) and 90% heptane + 10% ethanol [2-HEMA, 2-hydroxypropyl acrylate (2-HPA), cinnamal, and eugenol]. The flow rate was set at 0.5 ml/min, and the injection volume was 20 µl. The run time for each sample was 10 min. The presented HPLC method is based on a similar HPLC method developed by Goon et al. (2).

HPLC stock/standard solutions and calibration curves

Stock solutions were prepared from analytical standards by dissolving each standard in 99% heptane + 1% butanol (MMA) or 99% heptane + 1% ethanol (2-HEMA, 2-HPA, cinnamal, and eugenol). Standard solutions were formed by diluting stock solutions with an appropriate volume of 99% heptane + 1% butanol or 99% heptane + 1% ethanol. The standard solutions for each compound were used to make calibration curves, which were used for quantification and validation of the HPLC method. The correlation coefficients (R^2 -values) for the five allergens ranged between 0.9995 and 0.9999.

HPLC method validation

The analytical HPLC method was validated with regard to repeatability, level of detection (LOD), and level of quantification (LOQ). These parameters were determined by measuring the next-to-last standard solution in the calibration curves seven times in succession.



Fig. 1. Patch test chambers investigated in the present study (from left to right): IQ Ultimate™, IQ chamber™, and Van der Bend® transport container.

HPLC sample preparation

Samples weighing approximately 30 mg were taken from commercial patch test preparations and placed in the patch test chambers/containers. Each chamber was stored and analysed according to the following timetable: 0, 2 and 8 hr, and 24, 48, 72 and 7 days.

At the specified intervals, triple measurements were made from each patch test chamber. Approximately 25 mg of sample was weighed, placed in 25 ml or 1.5 ml volumetric flasks, and diluted in 99% heptane + 1% butanol (MMA) or 99% heptane + 1% ethanol (2-HEMA, 2-HPA, cinnamal, and eugenol). Finally, approximately 0.5 ml of solution was filtered, placed in 1 ml vials, and chromatographed.

2-HEMA was chosen for the investigation of homogeneity in a commercial patch test preparation. Approximately 30 mg of sample was weighed, placed in 10 individual 25 ml volumetric flasks, diluted in 99% heptane + 1% ethanol, and chromatographed.

Gas chromatography–mass spectrometry (GC-MS) instrumentation and chromatographic conditions

A Thermo Fisher Scientific GC-MS apparatus consisting of a Trace GC Ultra and a DSQ II mass spectrometer was used (Thermo Fisher Scientific, Waltham, MA, USA). A Zebron capillary gas chromatography (GC) column was fitted (length, 60 m; 0.25 mm internal diameter; film thickness, 0.25 μ m; temperature limits, minimum 20°C and maximum 250/260°C; Phenomenex). The injector was manual split/splitless and the mass spectrometer was used as a detector.

The chromatographic data were recorded and processed with XCALIBUR software (version 2.0.7; Thermo Fisher Scientific). In order to determine the various allergens in the chromatograms, we used authentic standards and the National Institute of Standards and Technology (NIST) 05 MS Search database and the NIST Automated Mass Spectral Deconvolution and Identification System 2.0 software.

The injection of stock/standard solutions was carried out with a 10 μ l injection syringe (SGE Analytical Science, Milton Keynes, UK). The headspace was removed with a 2.5-ml Gastight Headspace syringe (Hamilton, Bonaduz, Switzerland) and injected into the GC-MS apparatus.

The run time was set at 14.2 min, and the registered starting time of the mass spectrometer was 5 min. The initial temperature for the column oven was 35°C, and it was maintained for 2 min. Subsequently, the temperature was increased by 25°C/min for the following 8 min to a maximum temperature of 240°C. The maximum temperature was maintained for the last 4.2 min.

The manual injector temperature was set at 22°C in splitless mode (50 ml/min). The flow between the GC column and the mass spectrometer (left carrier) was 1.2 ml/min. The mass spectrometer detected the contact allergens with full scan (mass range: m/z 50–500) or single ion monitoring (SIM) (mass: m/z 131).

The above GC-MS method is completely new and was developed by the authors.

GC-MS stock/standard solutions and calibration curves

The stock and standard solutions for MMA and cinnamal were prepared in the same manner as previously described for HPLC. The correlation coefficients (R^2 -values) for MMA and cinnamal were 0.9972 and 0.9965, respectively. The injected volume of stock and standard solutions was 1 μ l.

GC-MS sample preparation

MMA and cinnamal were selected for the investigation of the evaporation process. The evaporation was analysed at 0 min, 20 min, 1 hr, 2 hr, and 3 hr. At each interval, quadruple measurements were made.

Samples of ~30 mg were taken from fresh commercial patch test preparations and placed in 20 ml headspace vials at room temperature. At the specified intervals, we removed the headspace (500 μ l) with a syringe, and analysed the headspace sample by GC-MS as described above. MMA and cinnamal were detected by full scan and SIM, respectively.

Results

Validation of the HPLC method

The detection and quantification limits were calculated, and verified experimentally. The LOQ of each allergen were verified by making standard solutions at the calculated LOQ concentration levels, and represented the last standard solution in each calibration curve. The LOD and LOQ results were as follows: 0.024 and 0.081 μ g/ml for eugenol, 0.0094 and 0.031 μ g/ml for cinnamal, 0.051 and 0.17 μ g/ml for 2-HEMA, 0.13 and 0.43 μ g/ml for 2-HPA, and 0.036 and 0.12 μ g/ml for MMA. The repeatability (RSD_r) for the five allergens was 2.73% for eugenol, 1.64% for cinnamal, 9.92% for 2-HEMA, 1.68% for 2-HPA, and 2.19% for MMA.

Analytical results for HPLC and GC-MS

The initial concentrations of acrylates/methacrylates and fragrance allergens found in commercial patch

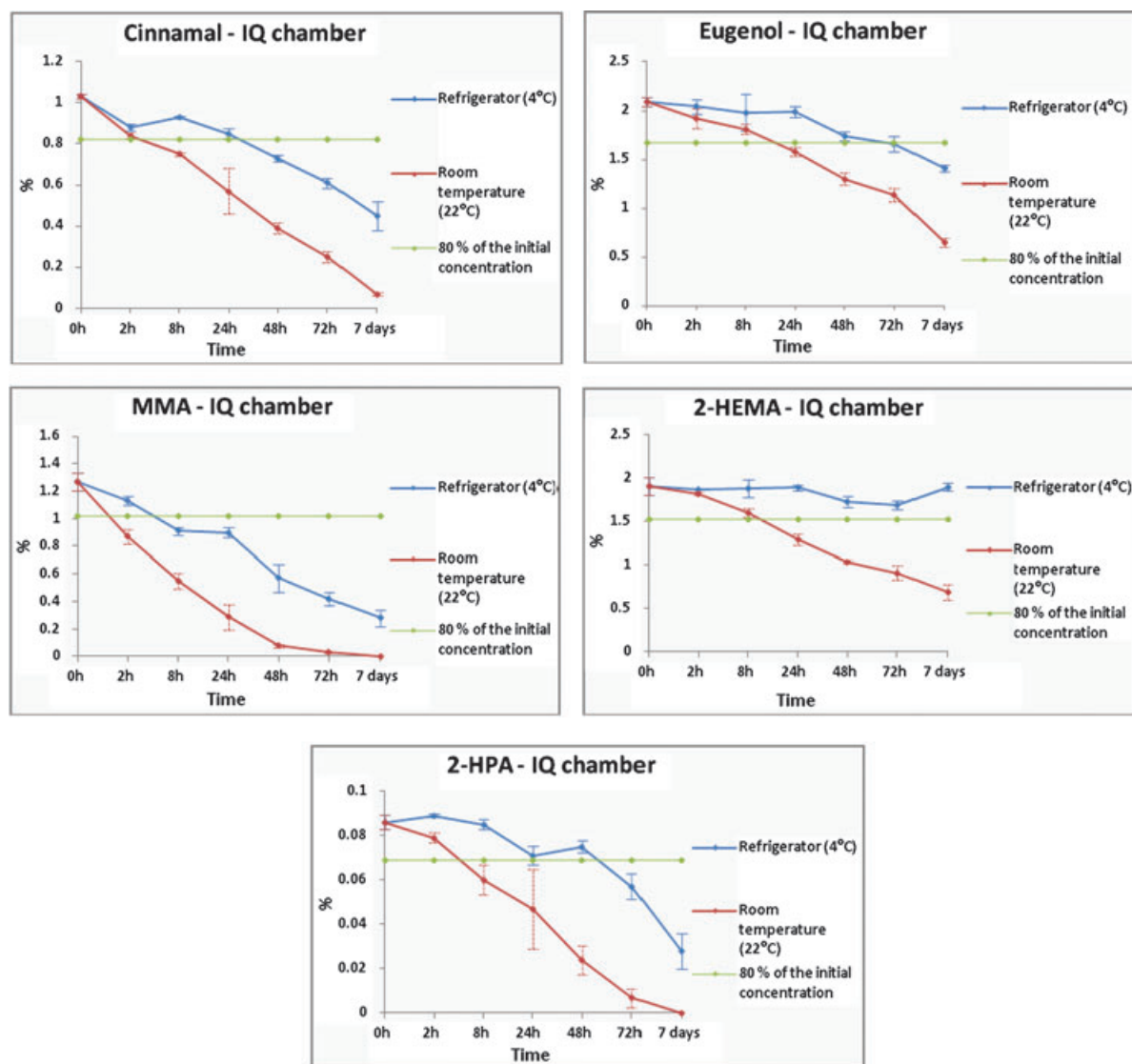


Fig. 2. The stability of the five allergens in the IQ chamber™ under different storage conditions. 2-HEMA, 2-hydroxyethyl methacrylate; 2-HPA, 2-hydroxypropyl acrylate; MMA, methyl methacrylate.

test preparations were determined at the first time interval, namely 0 hr. The results of the chemical analyses showed that the starting concentrations of acrylates/methacrylates were lower than stated by the producer, whereas the concentrations of fragrance allergens were in accordance with the stated concentrations (Figs. 2–4). According to the producer, the concentrations of methacrylates (MMA and 2-HEMA), acrylates (2-HPA), cinnamal and eugenol should have been 2%, 0.1%, 1% and 2% (wt/wt) petrolatum, respectively.

The initial concentrations of MMA, 2-HPA and 2-HEMA were only 1.27%, 0.086% and 1.91% (wt/wt), respectively.

The decrease in the concentration of acrylates/methacrylates was substantial when they were stored in the IQ chamber™ and IQ Ultimate™. In particular, MMA and 2-HPA showed a high degree of volatility in these patch test chambers, both at room temperature and in the refrigerator. 2-HEMA was likewise unstable at room temperature, whereas the initial concentration was maintained in the refrigerator.

MMA and 2-HPA showed decreases in concentration when stored in the Van der Bend® transport container under both storage conditions. The concentration of 2-HEMA was unchanged in the Van der Bend® transport container.

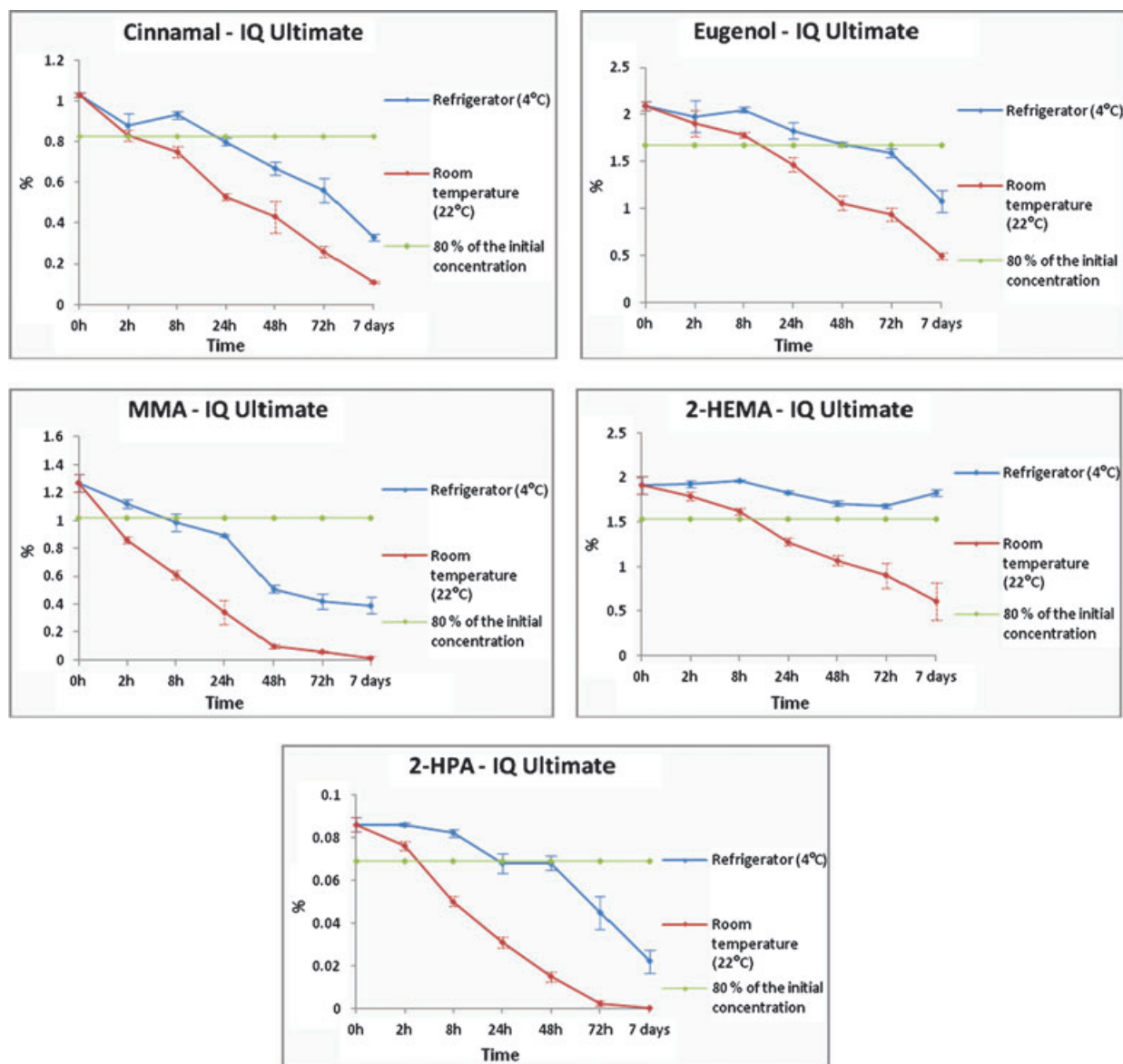


Fig. 3. The stability of the five allergens in the IQ Ultimate™ under different storage conditions. 2-HEMA, 2-hydroxyethyl methacrylate; 2-HPA, 2-hydroxypropyl acrylate; MMA, methyl methacrylate.

There was a pronounced decrease in the concentration of fragrance allergens in the IQ chamber™ and IQ Ultimate™ under both storage conditions. However, they showed stability in the Van der Bend® transport container.

In Figs. 2–4, the line denoting 80% of the initial concentration shows the selected stability limit. Generally, in the IQ chamber™ and IQ Ultimate™, the contact allergens dropped below the stability limit in the order MMA, cinnamal, 2-HPA, eugenol, and 2-HEMA, and in the Van der Bend® transport container, the contact allergens showed

acceptable stability under both storage conditions, except for MMA and 2-HPA, which required refrigeration. The largest standard deviations were found for MMA and 2-HPA.

The variability in concentration between 10 consecutive samples from a 2-HEMA commercial patch test preparation was acceptable. The mean concentration and standard deviation were 0.023 mg/ml and 0.0012 mg/ml, respectively.

The amount of MMA evaporating from pet. was larger than the amount of cinnamal evaporating from pet.

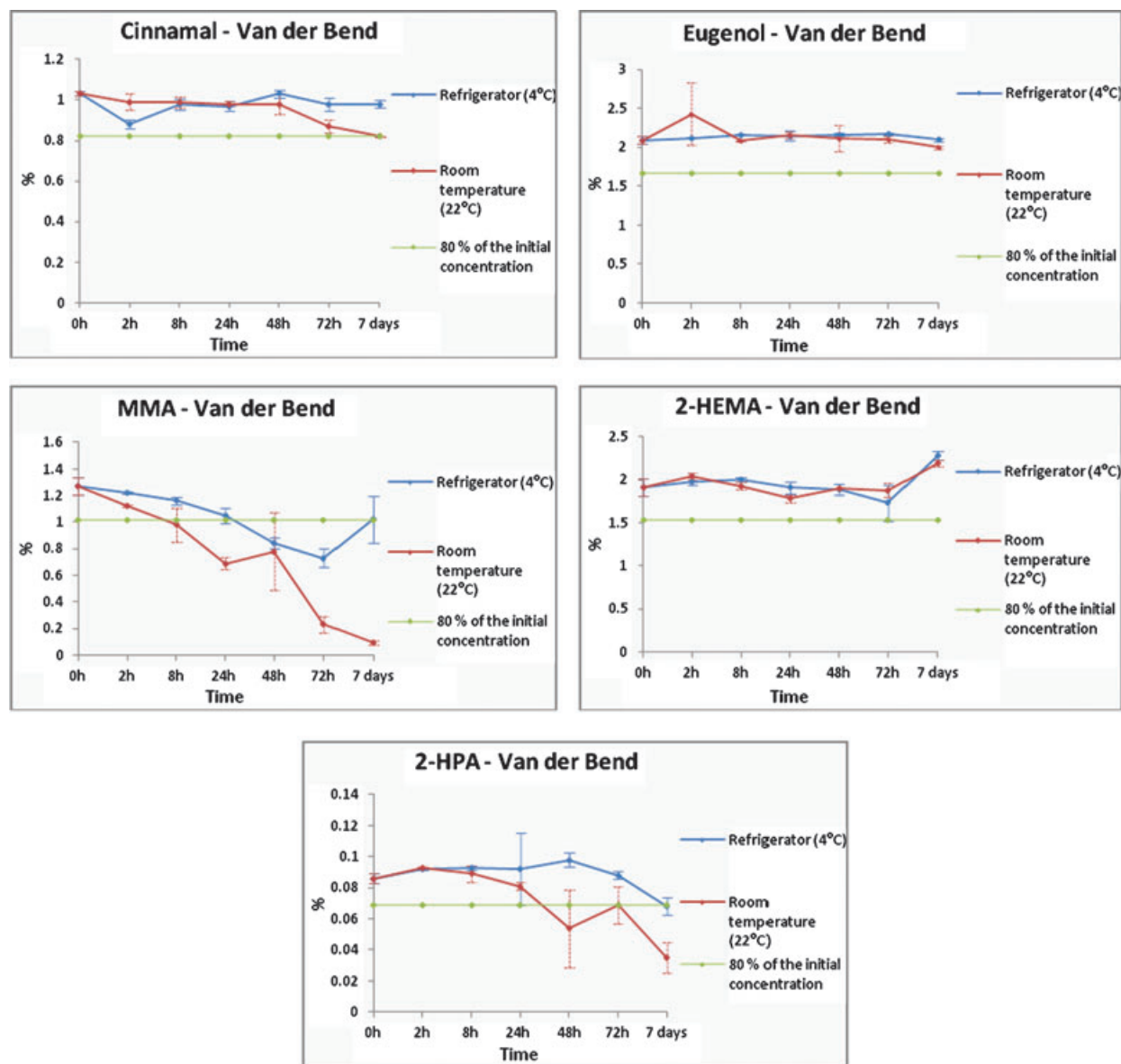


Fig. 4. The stability of the five allergens in the Van der Bend® transport container under different storage conditions. 2-HEMA, 2-hydroxyethyl methacrylate; 2-HPA, 2-hydroxypropyl acrylate; MMA, methyl methacrylate.

MMA evaporation continuously increased up to 2 hr, after which the amount of evaporation declined. This suggests that MMA was reaching equilibrium in the headspace. In contrast to that of MMA, the evaporation of cinnamal was constant.

Discussion

The term stability, in relation to commercial patch test preparations, has been defined, and a variation of $\pm 20\%$ in concentration has been chosen as acceptable prior to

the expiration date (7). This stability limit is controversial, because published results have demonstrated too low initial concentrations among fresh commercial patch test preparations (2–5). These findings are in accordance with the results in the present study. We presume that fresh commercial patch test preparations at patch test centres are being stored according to the recommendations given by the patch test suppliers. A possible solution to the stability problem is to shorten the shelf-life for volatile allergens.

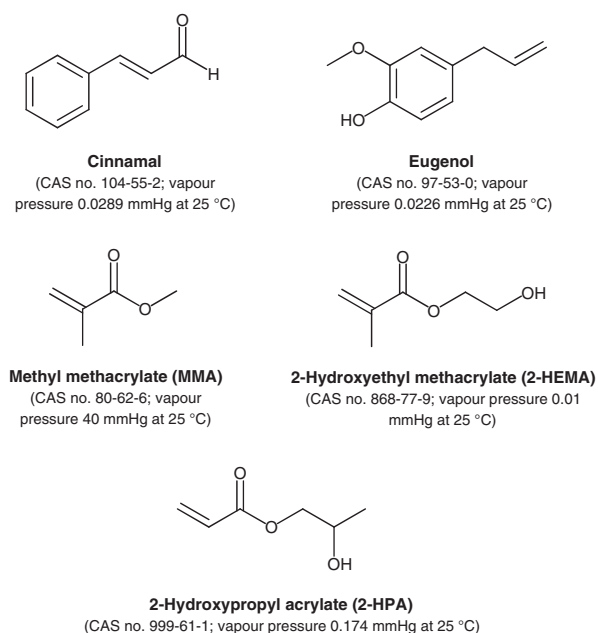


Fig. 5. Chemical structures of the five volatile contact allergens used in the present investigation.

To our knowledge, experiments have not been performed on the minimum concentration of methacrylate/acrylate in patch test preparations that needs to be present to elicit positive eczematous reactions. The recommended patch test concentrations for MMA and 2-HEMA are 2.0% (wt/wt) in pet., and that for 2-HPA is 0.1% (wt/wt) in pet. (8). However, the concentrations of MMA (1.27%), 2-HPA (0.086%) and 2-HEMA (1.91%) found in the present study may be acceptable from a clinical point of view. In the late 1980s, Kanerva et al. found that the concentrations of acrylates, at the time 0.5% (wt/wt) in pet. for certain acrylates, resulted in sensitized patients during routine patch testing (9). As a consequence, the concentrations of methacrylates/acrylates have been lowered whenever reports of patients being sensitized have been published (8). The inhomogeneity of allergen distribution in pet.-based test preparations is a well-known problem (6). In this study, 2-HEMA in pet. was homogeneous. However, one should be cautious in drawing conclusions on the basis of one test preparation. In this context, it should be mentioned that some producers add certain stabilizers and emulsifiers to the patch test preparations (2). Furthermore, it has been stated that certain allergens are being mixed with solvents prior to the mixing with pet. (7). These actions are considered to increase the stability of the patch test preparations. The larger standard deviations for

MMA and 2-HPA in this study may be attributable to inhomogeneity, and further studies are warranted.

In a few published studies, several explanations have been suggested for the loss of concentration among acrylates/methacrylates. These include chemical degradation (oxidation or hydrolysis), adsorption to surfaces, polymerization, evaporation, and suppliers' compounding errors (2, 5, 10). In our opinion, the most likely explanation is evaporation owing to the high vapour pressures of acrylates/methacrylates. The vapour pressures of MMA and cinnamal are 40 and 0.0289 mmHg at 25 °C, respectively (Fig. 5). As mentioned earlier, the amount of MMA evaporating from pet. was larger than that of cinnamal. This suggests that a higher vapour pressure will result in a higher rate of concentration decrease in pet. test preparations. In general, MMA showed the highest rate of decrease in the HPLC analyses, followed by 2-HPA, cinnamal, eugenol, and 2-HEMA. The vapour pressures decrease in the following order: MMA, 2-HPA, cinnamal, eugenol, and 2-HEMA (Fig. 5). It is quite clear that there is a correlation between vapour pressure and the rate of decrease in concentration. This correlation has also been shown in a previous study performed by Goon et al. (2).

The study design of the evaporation process is not entirely satisfying, because MMA almost reached equilibrium in the headspace. Nevertheless, the difference in evaporation between acrylates/methacrylates and fragrance allergens has been shown.

The affinity of allergens for various vehicles, such as alcohols and pet., may influence the stability of the patch test preparations. Many contact allergens mixed in pet. and stored in syringes at 4 °C are generally regarded as being stable for years (6). This might be true with regard to fragrance allergens, as well as methyl dibromoglutaronitrile (11), but it certainly does not apply for acrylates/methacrylates. This raises the question of whether pet. is the optimum vehicle for acrylates/methacrylates.

In conclusion, this study has shown that the Van der Bend® transport container is better at maintaining the stability of volatile contact allergen preparations than the IQ chamber™ and IQ Ultimate™. Moreover, the patch test chambers should be dosed shortly before testing of the patient.

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