

Evaluation of lymphocyte transformation tests as compared with patch tests in nickel allergy diagnosis

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Summary

Background. The patch test is considered to be the gold standard for diagnosing nickel (Ni) allergy. The lymphocyte transformation test (LTT) can also be used to detect Ni sensitization. However, little is known about the correlation between patch test and LTT reactions to Ni.

Objective. To establish and validate an LTT for Ni sensitization by comparison with patch test reaction and history.

Methods. Fifty individuals without self-reported 'Ni allergy' (controls) and 50 patients with self-reported suspicion of Ni allergy were included. A questionnaire-aided history was taken, and patch tests with at least the baseline series and LTTs with various NiSO₄ dilutions were performed.

Results. In the patch tests, 2 of 50 controls and 18 of 50 patients with self-reported suspicion of Ni allergy showed positive reactions to Ni. In the LTTs, 2 of 50 controls and 26 of 50 patients with self-reported suspicion of Ni allergy showed positive reactions to NiSO₄ 2.5 × 10⁻⁵ M, and 2 of 50 controls and 17 of 50 patients with self-reported suspicion of Ni allergy showed positive reactions to NiSO₄ 1 × 10⁻⁵ M. Sixteen of the 18 history-positive and patch test-positive patients (i.e. 88% sensitivity) were also LTT-positive, in contrast to only 2 of the 48 patch test-positive and history-negative patients (i.e. 96% specificity).

Conclusion. Performing the LTT with optimized stimulating conditions might be a useful additional tool for the diagnosis of Ni allergy if non-sensitized controls are included.

Key words: allergy; history; lymphocyte transformation test; nickel; patch test.

Nickel (Ni) contact allergy is common in the general population, with a prevalence of ~13% (1, 2). However, even a typical history is not always related to the respective allergy (3, 4). The reproducibility of patch test reactivity may decrease both after prolonged avoidance of allergen contact and in cases of weak positive reactions to Ni as the time since patch testing elapses (5). In a study by Ale and Maibach (6), simultaneous duplicate patch tests on opposite sides of the patients' back gave only 5% discordant

reactions (i.e. positive versus negative or doubtful). Thus, the authors stated that patch testing is a reasonably reproducible procedure as long as methodological error is minimized. In fact, patch testing is considered to be the gold standard for detection of contact allergy. Only in rare cases can patch tests not be performed, for example because of widespread eczema.

In attempts to develop *in vitro* tests for contact sensitization, the lymphocyte transformation test (LTT) (also known as the lymphocyte proliferation test) was developed in the 1960s to assess proliferative T cell reactivity to the antigen in question (7, 8). Subsequently, various assay modifications, such as different culture and stimulatory conditions or readout systems, were established (9–13). The LTT has proven to be a useful method to fill

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a diagnostic gap, for example in cases of suspected drug hypersensitivity (14, 15), and has also been adapted for *in vitro* detection of metal sensitization (16–19). In 1997, Cederbrant et al. still stated that the LTT would not be useful for the diagnosis of contact allergy to Ni, 'since a large number of false-positive results will be obtained' (20). Despite the growing number of LTT-related publications, there seems to be no general consensus on optimal assay conditions, but rather attempts of the various research groups to improve their individual laboratory approaches. We felt that optimization of LTT stimulatory conditions, by using a comparison between LTT and patch test reactions in the same patient, is largely missing. We thus assessed, in patients with a history of intolerance reactions to metallic objects potentially containing Ni, such as jewellery or jeans buttons, both LTT and patch test reactivity. In investigating the validity of LTT, we aimed to (i) exclude unspecific reactions by including non-sensitized controls, (ii) detect the best discriminatory stimulation condition for Ni-allergic individuals, and (iii) clinically validate the results by comparison with patch test results.

Methods

Patients

A total of 100 individuals who presented to our clinic for evaluation of suspected cutaneous allergy were sequentially enrolled in the study. Inclusion criteria were: no systemic immunosuppression, and the absence of any metallic orthopaedic-surgical implant or piercing. The participants consisted of two groups: 50 individuals (14 females, 36 males; mean age 53.50 ± 17.90 years) with no self-reported history of skin reaction upon metal contact (but suspected allergic or irritant dermatitis) as the control group; and 50 patients (46 females, 4 males; mean age 64.84 ± 8.92 years) with itching, erythema or eczema upon skin contact with metallic articles (and, correspondingly, suspected Ni allergy). After informed consent had been obtained, a questionnaire-aided medical history was taken. All patients were patch tested at least with the 29 allergens of the baseline series of the German Contact Dermatitis Research Group, and heparinized peripheral blood samples were taken from all participants. This investigation was approved by the local ethics committee. The general characteristics of the 100 participants of the study are shown in Table 1.

Questionnaire

The participants were asked a standardized series of questions regarding general history (such as smoking status,

current medication, and concomitant diseases) and a history focusing on skin changes such as redness, itching, eczema after contact with metallic articles (e.g. wrist-watches, jewellery, and jeans buttons), and allergic diseases already diagnosed. The presence of atopic diseases (atopic dermatitis, allergic rhinoconjunctivitis, and allergic bronchial asthma) was also noted. In addition, to firmly exclude this potential exposure factor, patients were asked twice about the absence of orthopaedic-surgical implants or piercing.

Patch testing

Patch testing was performed according to the guidelines of the German Contact Dermatitis Research Group (21). In every patient, at least the baseline series, containing 29 allergens, including Ni (5% pet.), chromium, and cobalt, was tested. Further test series were used according to exposure history. Test substances were applied on the upper back, and test readings were performed by physicians of the allergy unit on day (D) 2 and D3. The reactions reported here as positive were graded as +, ++, and +++. The respective results were entered into the Information Network of Departments of Dermatology (IVDK), www.ivdk.org, data collection program WinAlldat/IVDK.

Lymphocyte transformation test

Peripheral heparinized venous blood (60 ml) was obtained from all 100 individuals. In order to avoid boosting of cellular reactivity, blood samples were always taken prior to patch testing. Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation, and cultivated with the allergen and control substances. The LTT was performed according to Summer et al. (22, 23). Stimulation was performed in quadruplicate over a period of 6 days with the following substances: the T cell mitogen phytohaemagglutinin (PHA) 2.4 µg/ml (Biochrom, Berlin, Germany), and tetanus toxoid (TT) 5 µg/ml as control recall antigen (Chiron Behring, Berlin, Germany). Ni may be T cell-stimulatory in an unspecific fashion, in particular at high concentrations (16, 18, 24). Because, in our previous experiments, we had assessed the range of Ni dilutions with the best discriminatory *in vitro* response (22), we here used the low concentrations of 7.5×10^{-6} , 1×10^{-5} and 2.5×10^{-5} M NiSO_4 for stimulation. Unstimulated cells in medium alone were used as a control (14). After 5 days, cells were pulsed with [^3H]thymidine overnight, and proliferation was assessed according to the incorporated radioactivity. The results were expressed as the stimulation index (SI), which is determined from the ratio of incorporated radioactivity in

Table 1. General characteristics of the 100 individuals

	Control patients		Patients with suspected Ni allergy	
Age (years)	53.50 ± 17.90		64.84 ± 8.92	
Sex, n (%)	Male	Female	Male	Female
	36 (72)	14 (28)	4 (8)	46 (92)
Atopic disease, n (%)	20 (40)		27 (55.1)	

Control patients = individuals without history of cutaneous complications upon metal contact (i.e. no self-reported, suspected Ni allergy).

stimulated versus non-stimulated cultures. An SI of ≥ 3 was considered to indicate a positive reaction.

Statistical analysis

All data were analysed with IBM SPSS statistics 23.0 software. Statistical analysis was performed with the Wilcoxon–Mann–Whitney test, with significance set at $p < 0.05$.

Results

The most frequent indication for patch testing was contact dermatitis in both the individuals with and those without a history of metal (Ni) hypersensitivity. Twenty of the 50 control subjects and 27 of the 50 patients with self-reported suspicion of Ni allergy had reported the presence of atopic disease. This difference in the frequency of atopic diseases was not significant. Twelve of the 50 controls and 6 of 50 patients with self-reported suspicion of Ni allergy were smokers. The presence of dental implants was indicated by 4 of 50 controls and by 12 of 50 patients with self-reported suspicion of Ni allergy.

Patch test reactions

Of the 50 controls, 15 had one or more positive reactions to any of the allergens in the standard series. In terms of Ni reactivity, 48 had a negative patch test result and 2 had a positive patch test result. Of the 50 patients with self-reported suspicion of Ni allergy, 30 had reacted to one or more allergens of the baseline series, and 18 had a positive patch test reaction to Ni. These data are summarized in Table S1. Apart from Ni and cobalt, the list of the 10 allergens with the highest number of positive reactions was similar in the two groups (Tables 2 and 3).

Lymphocyte reactivity in the LTT

The LTT reactivity to Ni was evaluated with two approaches: first, in comparison with the patients' history of suspected Ni allergy; and second, by comparison with patch test reactions to Ni.

Table 2. The 10 most common allergens of the standard series in control individuals with no history of self-reported, suspected Ni allergy

Contact allergen	Positive reactions (n)	%
Fragrance mix II	6/50	12
<i>Myroxylon pereirae</i>	5/50	10
Methylchloroisothiazolinone/ methylisothiazolinone	5/50	10
Fragrance mix I	4/50	8
Colophonium	3/50	6
Methyldibromo glutaronitrile	3/50	6
Thiuram mix	2/50	4
Mercapto mix	2/50	4
Nickel(II) sulfate, 6H ₂ O	2/50	4
Epoxy resin	2/50	4

Table 3. The 10 most common allergens in patients with suspected Ni allergy

Contact allergen	Positive reactions (n)	%
Nickel(II) sulfate, 6H ₂ O	18/50	36
Fragrance mix I	8/50	16
<i>Myroxylon pereirae</i>	8/50	16
Propolis	4/50	8
Fragrance mix II	3/50	6
Cobalt(II) chloride, 6H ₂ O	3/50	6
Colophonium	2/50	4
Thiuram mix	1/50	2
<i>N</i> -isopropyl- <i>N'</i> -phenyl- <i>p</i> -phenylenediamine	1/50	2
Oil of turpentine	1/50	2

Baseline LTT reactivities of PBMCs of the controls and of the patients with a history of Ni hypersensitivity were comparable, as shown by the proliferative response to TT (mean SIs: 11.15 ± 1.09 and 11.61 ± 1.69 , respectively) and to the T cell mitogen PHA (mean SIs: 18.70 ± 1.53 and 21.43 ± 2.26 , respectively). LTT reactivities to Ni were as follows. With NiSO₄ 2.5×10^{-5} M, 2 of the 50 controls were positive and 48 were negative, and of the 50 patients with self-reported Ni allergy, 26 were positive and 24 were negative. With NiSO₄ 1×10^{-5} M, 1 of the 50 controls was positive, and 49 were negative, and in the group

with self-reported Ni allergy, 17 were positive and 33 were negative. With NiSO_4 7.5×10^{-6} M, 1 of the 50 controls was positive and 49 were negative, and in the group with self-reported Ni allergy, 11 were positive and 39 were negative. These data are summarized in Fig. 1.

On the basis of positive (20 individuals) or negative (80 individuals) patch test reactions irrespective of history, we observed that 17 of 20 patients with positive patch test reactions also had a positive LTT response to at least one of the Ni dilutions. Of the 80 patch test-negative individuals, 67 also had a negative Ni LTT reaction but 13 had a positive Ni LTT reaction (Fig. 2). As shown in Fig. 2a–c, the extent of LTT reactivity (the SI) in patch test-positive patients was also related to the strength of the patch test reaction (graded from + to +++).

When history was included, we found the following:

- (1) The LTT response in the 48 patch test-negative/50 history-negative controls was positive in only 2 individuals to NiSO_4 2.5×10^{-5} M and in 0 both to NiSO_4 1×10^{-5} M and to 7.5×10^{-6} M.
- (2) The LTT response in the 18 patch test-positive/history-positive patients was positive in 12 individuals to NiSO_4 2.5×10^{-5} M, in 8 to NiSO_4 1×10^{-5} M, and in 5 to NiSO_4 7.5×10^{-6} M. When reactivity to both NiSO_4 2.5×10^{-5} M and 1×10^{-5} M was considered, 16 of the 18 patch test-positive/history-positive patients reacted positively.
- (3) The LTT response in the 32 patch test-negative/history-positive patients was nevertheless positive in a substantial proportion of the patients, that is, in 13 to NiSO_4 2.5×10^{-5} M, in 9 to NiSO_4 1×10^{-5} M, and in 6 to NiSO_4 7.5×10^{-6} M.

Thus, taken these findings together: (i) stimulation with NiSO_4 2.5×10^{-5} M and 1×10^{-5} M gave the best discriminatory results when the patch test was used as a reference; and (ii) there was additional LTT reactivity in history-positive/patch test-negative patients as compared with the controls (Fig. 1).

Sensitivity and specificity of the LTT as compared with the patch test

To make direct comparisons, we used the patch test outcome and the combined LTT reaction resulting from stimulation with NiSO_4 2.5×10^{-5} M and 1×10^{-5} M (i.e. a positive reaction to one or both concentrations was rated as 'overall positive'). As 16 of 18 patch test-positive/history-positive individuals were also LTT-positive, the sensitivity was 88%. Of the 48 patch test-negative/history-negative individuals, 2 had

a positive LTT reaction (to NiSO_4 2.5×10^{-5} M), so the specificity was 96%. It is an open question whether the LTT may have higher actual sensitivity, as a substantial proportion of history-positive/patch test-negative patients reacted in the LTT.

Discussion

Allergic reactions resulting from cutaneous Ni exposure and its classic manifestation (i.e. allergic contact dermatitis) are usually considered to reflect delayed-type (type IV) hypersensitivity. Within the general population in Europe, approximately 10–15% of females and 2–5% of males are Ni-allergic (1, 2). These numbers will probably not decrease dramatically, as exposure to Ni-releasing articles is continuing, despite Ni regulation (25, 26). The patch test as an *in vivo* test for the detection of T lymphocyte-mediated sensitization is considered to be the 'gold standard' (27). However, several restrictions reduce the accuracy of patch testing: the results are not 100% reproducible at synchronous testing (4, 6), and season, menstrual cycle, epidermal barrier, metal implant status and the timing of test readings may influence the results (6, 28–31). In fact, when simultaneous duplicate patch tests have been performed on opposite sides of the upper back, wide variations in reproducibility have been observed. For example, Gollhausen et al. reported that 2 of 4 patch test reactions to Ni were non-reproducible (although only 35 patients were investigated) (4), whereas Ale and Maibach reported, in their study on 500 consecutive patients, that, of the 435 positive patch test reactions, only 22 (5%) were discordant on simultaneous patch testing on both sides of the upper back (6). Uter et al., in view of the conflicting evidence concerning seasonal variability, reported on a large clinical sample analysed by linking meteorological/seasonal factors with patch test reactivity (29). However, only for formaldehyde was a distinct variation observed. Ni is also known for its ability to give late patch test reactions (31–34). We recently reported a gain of 34% positive Ni patch test reactions in 250 patients (i.e. 21/250 versus 32/250) by use of an additional D6 reading (34). Accordingly, some limitations apply to the data presented here. First, the patients reported here had patch tests read only until D3; second, no serial dilutions of Ni patch tests were applied. As the patch test may have limitations in detecting sensitized patients, the LTT was developed as additional tool to identify lymphocyte sensitization in putative allergic clinical reactions. The use of the LTT as an *in vitro* assay for evaluation of lymphocyte reactivity was first published in the 1960s (7, 8, 35). Since then, the LTT has often been performed, with varying results. Particularly in the diagnosis

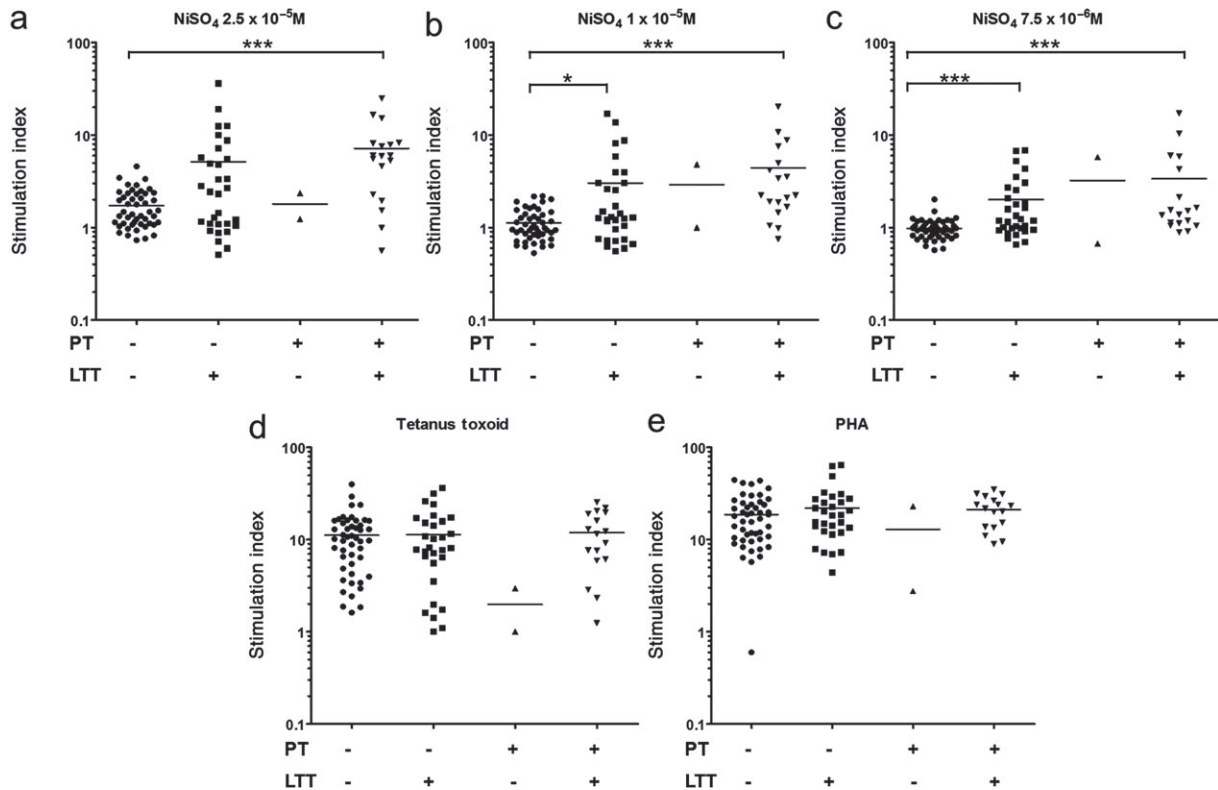


Fig. 1. (a–e) Relationship between lymphocyte transformation test (LTT) reactivity and patch test (PT) reactivity in the 100 individuals. Tetanus toxoid was the control recall antigen; phytohaemagglutinin (PHA) was the control T cell mitogen. $*** p < 0.001$.

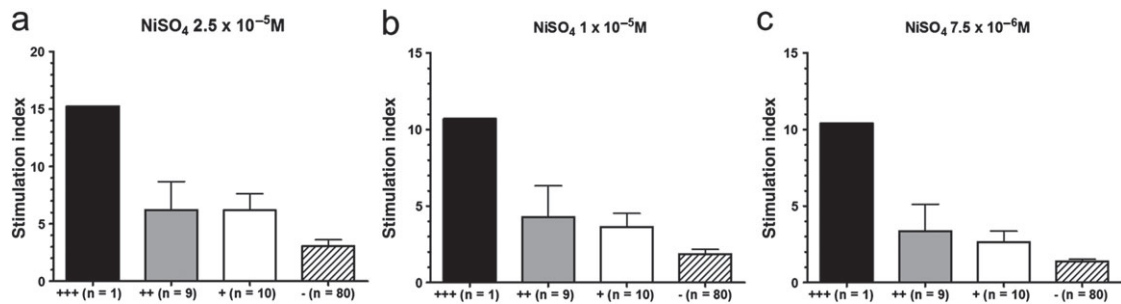


Fig. 2. (a–c) Lymphocyte transformation test reactivity to the three Ni concentrations [(a) NiSO_4 $2.5 \times 10^{-5} \text{ M}$, (b) NiSO_4 $1 \times 10^{-5} \text{ M}$, and (c) NiSO_4 $7.5 \times 10^{-6} \text{ M}$] in patch test-positive and patch test-negative individuals (in total, 100 individuals) irrespective of history.

of drug hypersensitivity, several authors reported encouraging experience with the LTT (13–15, 36, 37). Nyfeler and Pichler (15) retrospectively analysed 923 patient files – the majority of positive (drug-allergic) cases being caused by β -lactams – for their LTT results. They reported that the LTT used under optimized conditions, in analogy to Trautmann et al. (36), increased the detection rate to a sensitivity of 78% and specificity of 85%.

A comparison with patch test reactivity confirms the validity of the LTT results. Thus, we used patch test

reactivity to Ni as a reference in our 100 patients – but further divided these patients into ‘history-negative’ and ‘history-positive’. Furthermore, the presence of metallic potentially Ni-releasing orthopaedic-surgical implants or piercings was avoided to exclude the possibility of these potentially interfering with LTT reactivity (38). We found that optimized culture conditions and reference to two Ni stimulatory concentrations (NiSO_4 $1 \times 10^{-5} \text{ M}$ and $2.5 \times 10^{-5} \text{ M}$) showed the best concordance between patch test and LTT reactivity (88%), with the lowest

proportion of non-specific reactions being seen in patch test-negative/history-negative individuals (specificity of 96%). A similar test evaluation against the patch test was reported both by Spoerri et al. (39), using a non-radioactive flow cytometry assay including patch test-negative controls, and by Pacheco et al. (40), who introduced a peak SI in controls for calibration. Our approach differs in two respects. First, we considered the combined use of two *in vitro* Ni stimulatory concentrations, that is, PBMC positive reactivity to both or at least one of them, as a more suitable approach. Second, we included the history of metal intolerance as further information, and found that, in patients with a positive history concerning Ni allergy, the LTT may point to a number of Ni-allergic cases undetected by patch testing. Several confounding factors might, however, be considered. As mentioned above, we did not perform a late patch test reading, so that a proportion of Ni patch test reactivity may have been missed. In addition, many cases of 'self-reported Ni allergy or rash from metal items in skin contact' may not be true Ni allergy. Josefson et al. (3) showed rather low validity of self-reported Ni allergy, and pointed out that 'Ni allergy was verified in only 59–60% of those who reported Ni allergy'. Correspondingly, in their study, women with a history of childhood eczema had a higher number of false-positive answers. Possibly, patients with atopic skin would more easily develop irritant reactions to metals (3, 41). However, the moderate difference in atopy frequency in our two groups would not explain the higher proportion of LTT reactivity in history-positive/patch test-negative individuals.

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Higher concentrations of NiSO₄ for *in vitro* stimulation might be unspecifically mitogenic, as already discussed by Kimber et al. (42) and by Cederbrant et al. (16). In 2010, the research group of Goebeler identified a unique Ni²⁺-responsive region in human toll-like receptor 4 (24). This implies that Ni²⁺ may directly induce proinflammatory gene expression. Thus, we tried to minimize such bias by using lower dilutions to keep 'unspecific' reactivity as low as possible. This is also reflected by Ni LTT reactivity showing a correlation with the strength of patch test reactions. Finally, the LTT with the control antigen TT and the mitogen PHA showed similar response patterns in our two patient groups, indicating the good comparability of PBMC reactivity.

In summary, our results indicate that LTT optimization with assay calibration based on patch test-negative/history-negative controls helps to detect Ni-sensitized individuals. Patients with Ni reactivity diagnosed by the patch test are identified with high accuracy by such an optimized LTT. It is thus also a valuable tool in cases where a patch test cannot be performed. In addition, it might be useful to analyse history-positive/patch test-negative patients and patients sensitized to Ni by internal exposure, for example patients with metal implants.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. PT results for the allergens of the standard series. Control patients = individuals with no self-reported, suspected Ni allergy.

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Table S1: PT results to the allergens of the standard series. Control patients = individuals with no self-reported, suspected Ni allergy.

Contact allergen	Control patients (positive reaction) n = 50	%	Patients with suspected Ni allergy (positive reaction) n = 50	%
Potassium dichromate	1	2	0	0
Thiuram mix	2	4	1	2
Cobalt (II)-chloride, 6H ₂ O	1	2	3	6
Balsam of peru	5	10	8	16
Colophonium	3	6	2	4
N-Isopropyl-N`-phenyl- p-phenylenediamine	0	0	1	2
Lanolin alcohol	1	2	0	0
Mercapto mix	2	4	0	0
Epoxy resin	2	4	0	0

Nickel (II)-sulfate, 6H ₂ O	2	4	18	36
p-tert.-Butylphenol formaldehyde resin	0	0	0	0
Formaldehyde	1	2	0	0
Fragrance mix	4	8	8	16
Turpentine	1	2	0	0
(Chloro)-Methyl Isothiazolinone (MCI/MI)	5	10	0	0
Parabens	0	0	0	0
Cetyl stearyl alcohol	0	0	0	0
Zinc- diethyldithiocarbamate	1	2	0	0
Dibromdicyanobutane	3	6	1	2
Propolis	0	0	4	8
Bufexamac	0	0	1	2

Compositae mix	0	0	0	0
Mercaptobenzothiazol	0	0	0	0
Lyrar	2	4	0	0
Bronopol (2-Bromo-2-nitropropan-1,3-diol)	1	2	0	0
Fragrance mix II	6	12	3	6
Ylang Ylang (I + II) oil	0	0	0	0
Sandal wood oil	0	0	1	2
Jasmine absolute	2	4	1	2