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Stability control of valerian ground material and extracts: a new HPLC-method for the routine quantification of valerenic acids and lignans

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A new HPLC-method for the separation of medium polar and nonpolar compounds in preparations of *Valeriana officinalis* was established for stability control. Powdered valerian root and a commercial ethanolic valerian extract were investigated for apparent differences in stability behaviour. Storage conditions were chosen according to the ICH-guidelines. Changes in composition of valerenic acids and lignans were observed depending on storage conditions and packaging materials. Hydroxyvalerenic acid, pinoresinol and hydroxypinoresinol were identified as degradation products in Valerian *root*, especially during accelerated testing. Ethanolic extracts appeared not to be as sensitive for chemical degradation under climatic influences compared to the crude plant material, and showed no increase in the amounts of lignan-aglyka. In comparison, extracts showed high sensitivity on changes of physical properties like loss on drying and viscosity.

1. Introduction

Stability of herbal drugs, drug preparations and finished products is more difficult to monitor as for chemical defined monosubstances due to the complex composition of the matrix. The entire herbal drug or the extract stands for the active drug substance, and is been treated in quality control like single chemical entities (CPMP 2002). For most of the herbal drugs no stability data are documented in Pharmacopoeia monographs, so that for many plants validated results are required.

Herbal medicinal products of *Valeriana officinalis* L. (valerian) like extracts or tea preparations etc. belong to the most frequently used mild sedatives on the German market. Consequently, this drug was chosen to determine the stability of its constituents and to identify the most important degradation products under the influence of different environmental conditions.

Although the major compounds of valerian have been isolated and pharmacologically investigated, the molecular mechanisms of the sedative action is still a matter of discussion. If constituents with known therapeutic activity are unknown, marker compounds, which should be specific for the herbal drug, like valerenic acid, have to be used for quality surveillance (Lazarowych and Pekos 1998). It has to be verified that markers remain within distinct specification limits under the recommended storage conditions. It must also be shown by means of appropriate fingerprint methods, that other substances in the herbal drug preparation are likewise stable (CPMP 2002). A typical ''stability-analysis'' is established by chromatographic fingerprint methods like thin layer chromatography, gas chromatography and high performance liquid chromatography, especially with diode array detection (Lazarowych and Pekos 1998).

The aim of the presented work was to observe changes in the chemical composition of specific valerian marker compounds like valerenic acids and lignans with a new and more efficient HPLC-method to reveal possible degradation pathways. The design of the stability testing was based on the ''Note for guidance on stability testing: stability testing of existing active substances and related finished products'' (CPMP 1998). As recommended, storage conditions of 25 °C/60% relative humidity, 30 °C/60% rh and 40 °C/75% rh were established. To compare the influence of the packaging material, the extracts were stored in polyethylene(PE)-wide neck bottles, sealed aluminium bags and PE-bags, for simulation miniaturised industrial container systems. An unpackaged extract was analyzed as well.

2. Investigations, results and discussion

2.1. A new reversed-phase HPLC-gradient system

Valerenic acids and valepotriates have already been subjected to various HPLC systems (Hänsel and Schulz 1982; Gorbato and Lolla 1996), which were used as a starting point for a new separation in order to detect more polar constituents. The amount of water at the beginning of the gradient system was increased and the duration of development extended. Fig. 1 shows the resulting HPLC-fingerprint chromatogram of an extract of powdered valerian root. The individual assignement to valerenic acids and lignans was done by spectrophotometry (UV-DAD-detektion). Furthermore, as indicated in Table 1, some of the compounds were isolated and identified by electrospray ionisation mass spectrometric and proton nuclear magnetic resonance. The linearity of the detectors response was checked

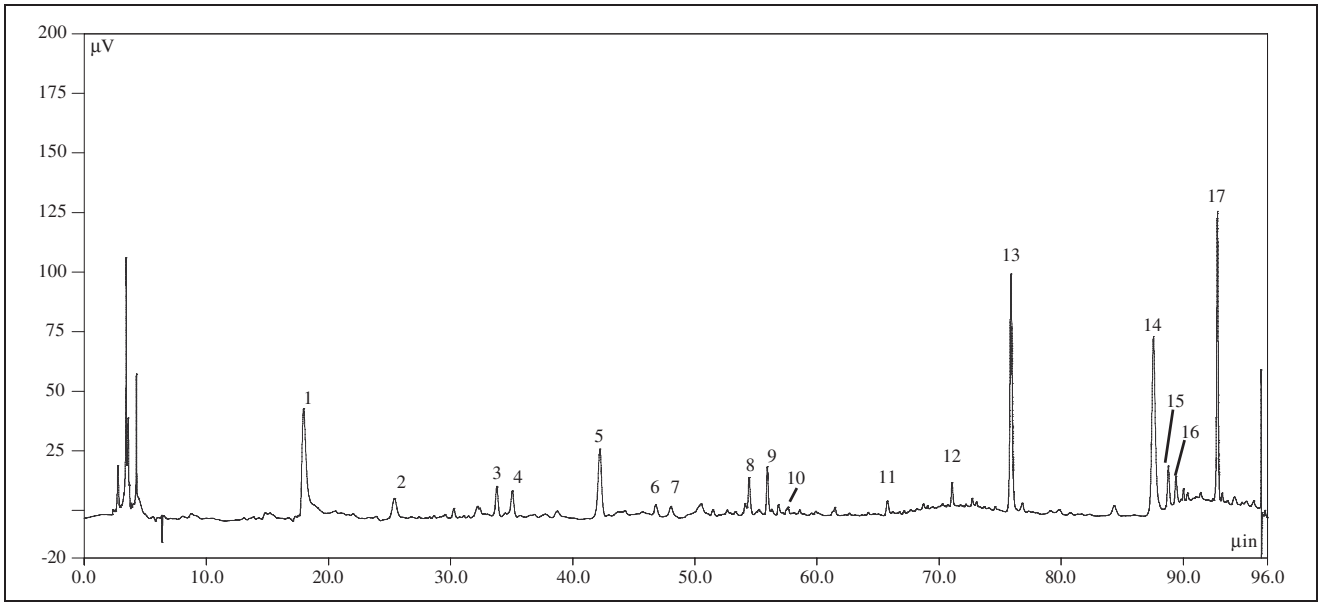

Fig. 1: Typical reversed phase HPLC-fingerprint of Valerian root extract

Table 1: Peak assignement of identified compounds from *Valeriana officinalis*

Peak-Nr.	Retention time (min)	Identity	MW	ESI neg.m/z $[M-H^+]^-$	UV-Maxima λ_{max} (nm)	Type of compound
1	17.96	not defined				
2	25.41	not defined				
3	33.78	not defined			225, 277	Lignan[4]
4	35.05	not defined			225, 277	Lignan[4]
5	42.22	Pinoresinol-diglucoside[2,3]	682	681.3	225, 276	
6	46.8	not defined			225, 277	Lignan[4]
7	48.05	Hydroxypinoresinol-glucoside[2]	536.2	535.2	227, 277	
8	54.45	not defined			227, 277	Lignan[4]
9	55.94	not defined				
10	57.72	Hydroxypinoresinol[2]	374	373.1	227, 277	
11	65.76	Pinoresinol[2,3]	358	356.9	230, 279	
12	71.06	Hydroxyvalerenic acid[2]	250	248.9	219	
13	75.89	Acetoxyvalerenic acid[2]	292	291.0	217	
14	87.55	Valerenic acid[1]	234	232.9	217	
15	88.78	not defined			254	Valepotriate[4]
16	89.39	not defined			254	Valepotriate[4]
17	92.79	Valerenal[4]			229	

[1] identified by coelution with standard-references
[2] identified by ESI-MS after isloation
[3] identified by ^{1}HNMR after isolation see 3.7
[4] Classification to compound groups by comparison with the UV-spectra

for valerenic acid and pinoresinol. Values for regression are reported in Table 2. The standard deviation of area repeatability after injection of 4 samples was calculated with 1.22% for valerenic acid and 0.99% for pinoresinol.

2.2. Variations of compounds in Valerian root samples

The powdered *Valeriana* root showed distinct changes in chemical composition depending on storage conditions. Figs. 2–5 represent the appropriate ratios of valerenic acids during a 500 day storage period. Considering only the changes in total content on sesquiterpenic acids expressed as valerenic acid, as described in Fig. 2, a 30% decrease was documented independent of environmental influences. This value, however, is still high enough to fulfill the demands of the pharmacopoeia of 0.17% content on sesquiterpenic acids (Ph.Eur. 2002a). After closer examination differences were recognized in degradation of the three valerenic acids depending on the respective storage conditions. Valerenic acid is slightly sensitive against environmental influences as seen in Fig. 3. The total amount decreases to 80% of the initial value under all climatic conditions. Compared to va-

Table 2: Validation parameters of the established HPLC-method

	Regression equation y (µg/ml) =	Correlation coefficient	LOD (ng)	Linearity area (ng)
Valerenic acid	36.86 × PA	0.9989	200	300–1800
Pinoresinol	11.2 × PA	0.9999	120	300–7200

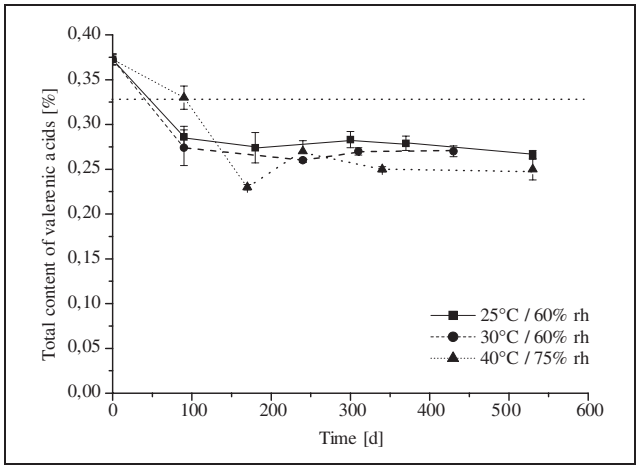

Fig. 2: Decrease of total content of valerenic acids (sesquiterpenic acids) in Valerian root depending on storage conditions (dotted line represents a 10% loss on initial value; ± values represent SD for n = 4)

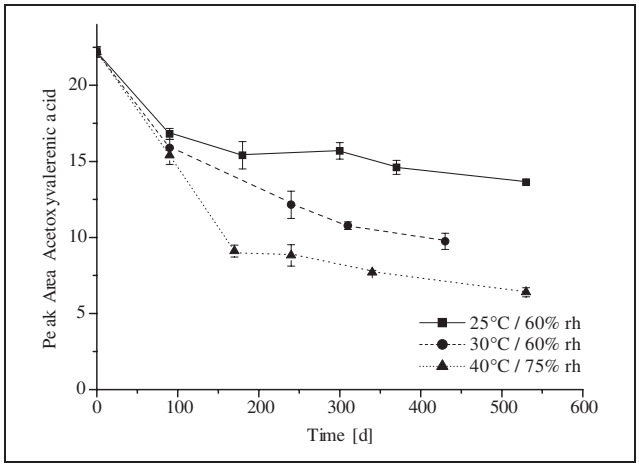

Fig. 4: Decrease of acetoxyvalerenic acid in Valerian root depending on storage conditions

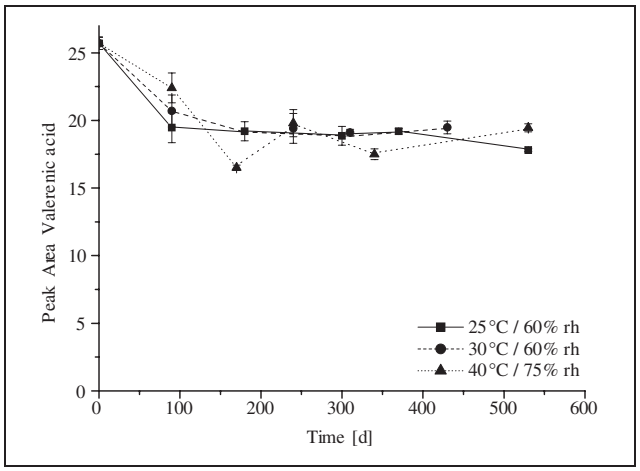

Fig. 3: Decrease of valerenic acid in Valerian root depending on storage conditions

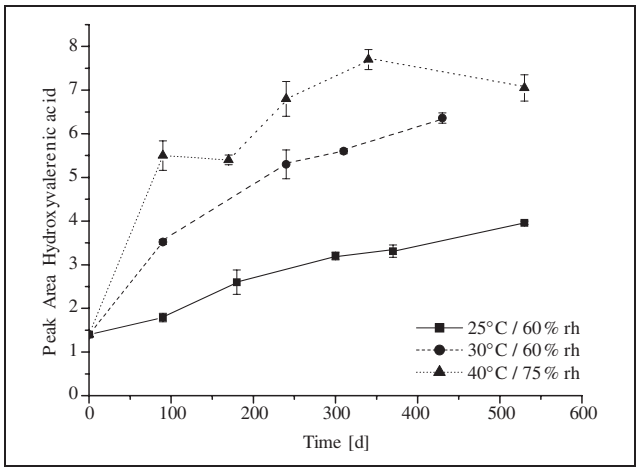

Fig. 5: Increase of hydroxyvalerenic acid in Valerian root depending on storage conditions

lerenic acid, the acetoxyvalerenic acid showed severe degradation, depending on the storage conditions (Fig. 4) with maximal degradation of 70% loss on content at 40 °C/ 75% rh. Hydroxyvalerenic acid increased since it is known as the degradation product of acetoxyvalerenic acid to five times more of the initial Peak Area under the same storage conditions. Even at 25 °C/60% rh the increase amounted to 180% of the initial value (Fig. 5).

Based on this facts Fig. 6 shows that conclusions on stability would mislead if the development of the single substances is disregarded. The sum of valerenic acids has decreased on the same level for all tested climatic conditions. The rate of hydroxyvalerenic acid is increased in parallel to the material stressed under the climatic conditions. With 23.7% rate of total sesquiterpenic acids content at 40 °C/75% rh the value comes to ten times more

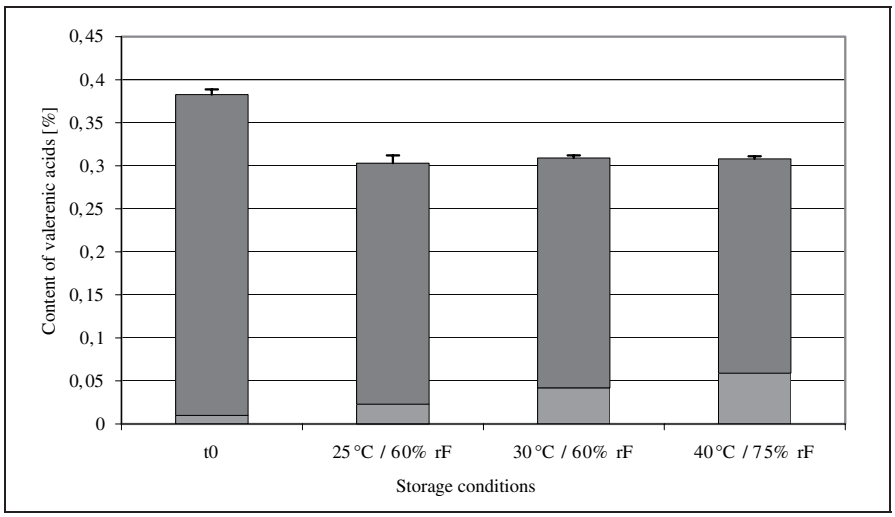

Fig. 6:
Comparison of hydroxyvalerenic acid (grey area) ratio on total sesquiterpenic acid content (squared area) after one year of storage under defined climatic conditions in Valerian root

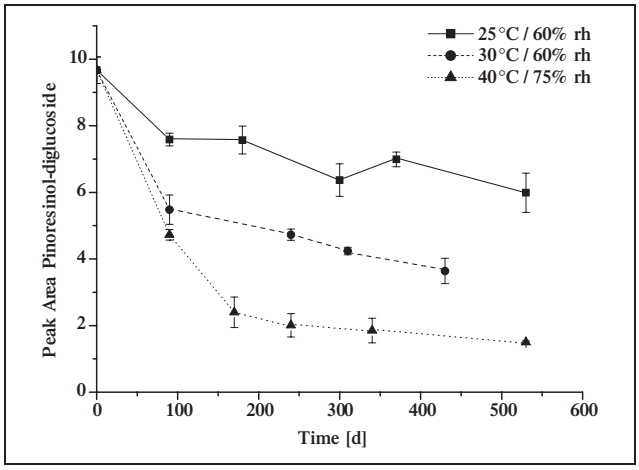

Fig. 7: Decrease of pinoresinol-diglucoside in Valerian root depending on storage conditions

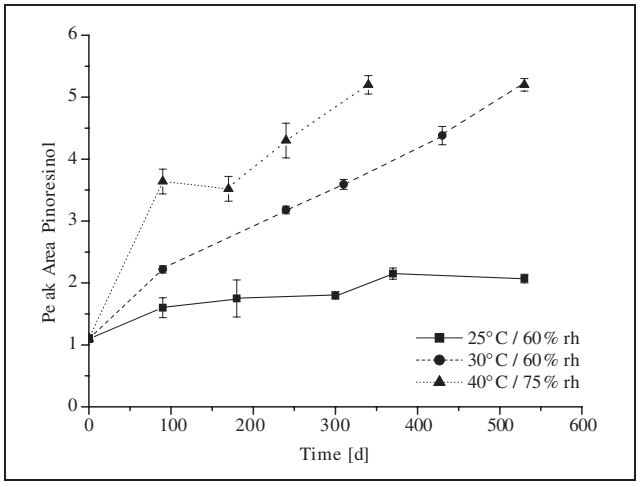

Fig. 8: Increase of pinoresinol in Valerian root depending on storage conditions

than the initial share, compared with 8.2% and 15.7% at 30 °C/60% rh and 75% rh.

Furthermore, significant transformations occurred within the fraction of the lignan compounds. The hydrolysis of lignan-glycosides like pinoresinol-diglucoside (Fig. 7) led to an increase of aglyca like pinoresinol (Fig. 8) and hydroxypinoresiol (Fig. 9). As shown for the valerenic acid

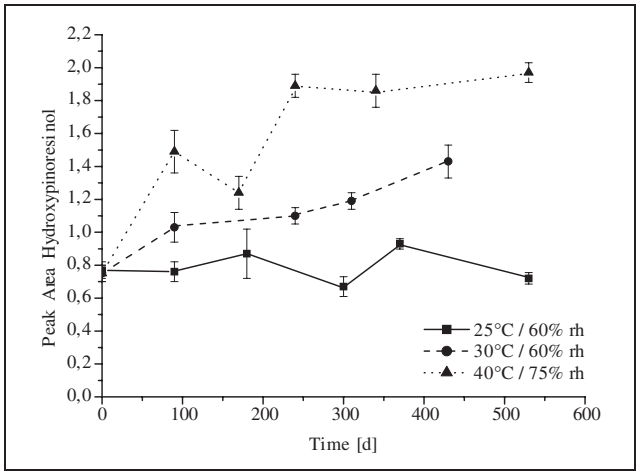

Fig. 9: Increase of hydroxypinoresinol in Valerian root depending on storage conditions

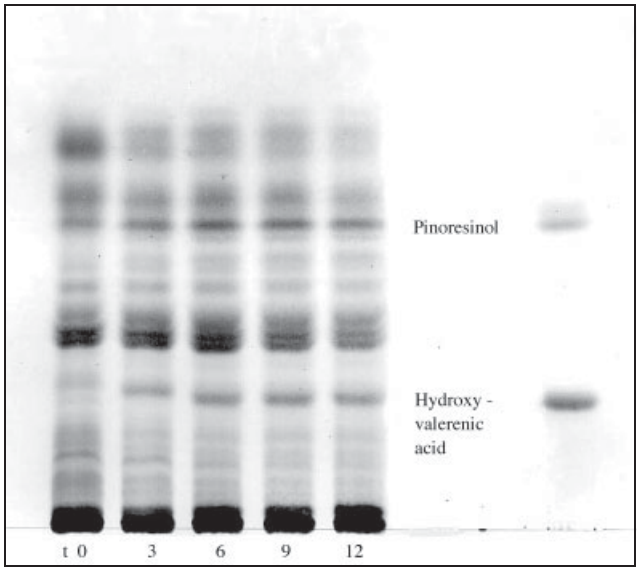

Fig. 10: TLC-profiling of the powdered drug material stored at 40 °C/75% rh.; (values at the bottom represent the months of storage)

esters, the intensity of the hydrolysis strongly depends on the storage conditions.

Finally it was examined, if the increase of degradation products possibly can be detected by TLC. An existing method for the separation and isolation of lignans (Bodesheim 1996) was modified as a stability indicating analytical method. Comparison of samples stored at 40 °C/75% rh for 12 months showed also in TLC the above described increase of pinoresinol and hydroxy valerenic acid (Fig. 10).

2.3. Variations of compounds in ethanolic valerian extracts

In a second step it was investigated if the showed stability results are identical and can be confirmed for a commercial valerian extract. Apart from changes in the typical chemical composition depending on storage conditions, the influence of packaging material was also examined. Figs. 11–16 show the decrease of acetoxyvalerenic acid and the increase of the corresponding hydroxyvalerenic acid and the reliance on the respective storage and packaging forms. PE wide neck bottles and sealed aluminium bags under all conditions showed negligible changes. However, extracts in PE-bags and unpackaged extracts already at of 30 °C/60% rh proved to have a distinct in-

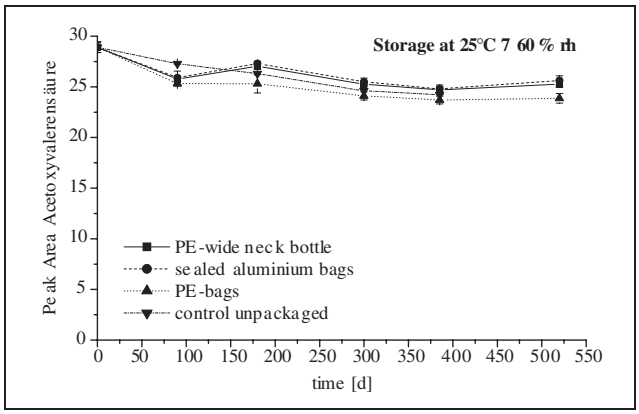

Fig. 11: Decrease of acetoxyvalerenic acid in ethanolic valerian extract in different packaging forms at 25 °C/60% rh

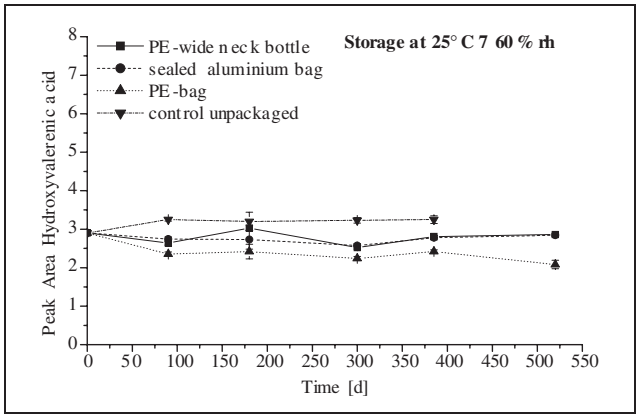

Fig. 12: Hydroxyvalerenic acid in ethanolic valerian extract in different packaging forms at 25 °C/60% rh

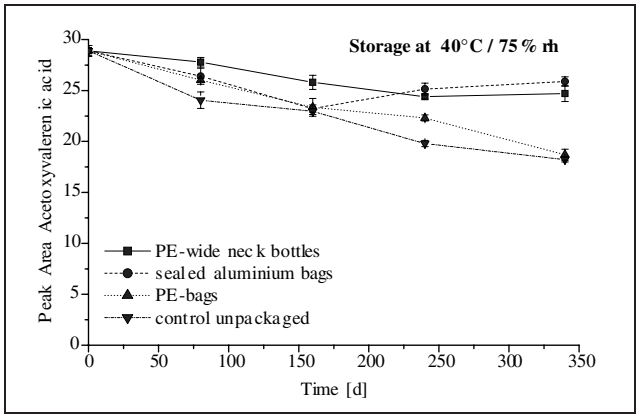

Fig. 15: Decrease of acetoxyvalerenic acid in ethanolic valerian extract in different packaging forms at 40 °C/75% rh

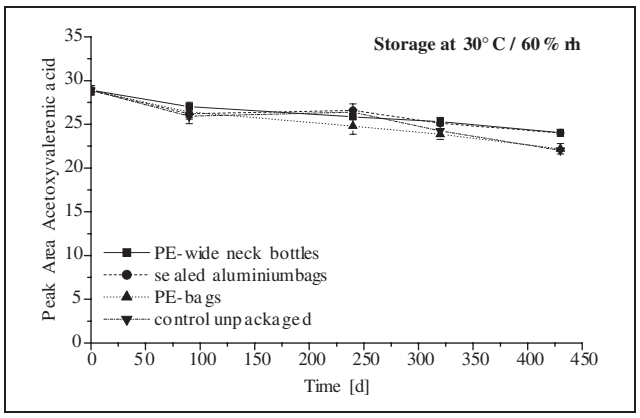

Fig. 13: Decrease of acetoxyvalerenic acid in ethanolic valerian extract in different packaging forms at 30 °C/60% rh

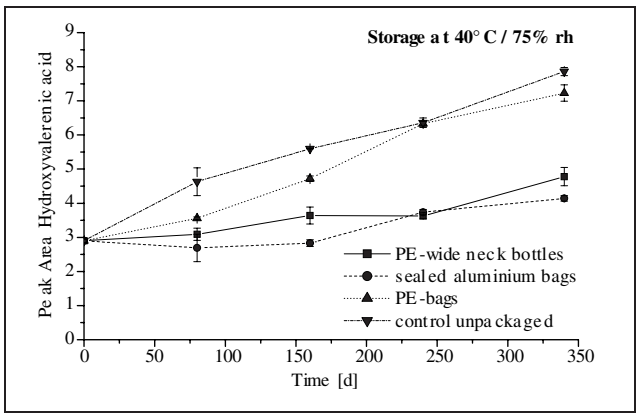

Fig. 16: Increase of hydroxyvalerenic acid in ethanolic valerian extract in different packaging forms at 40 °C/75% rh

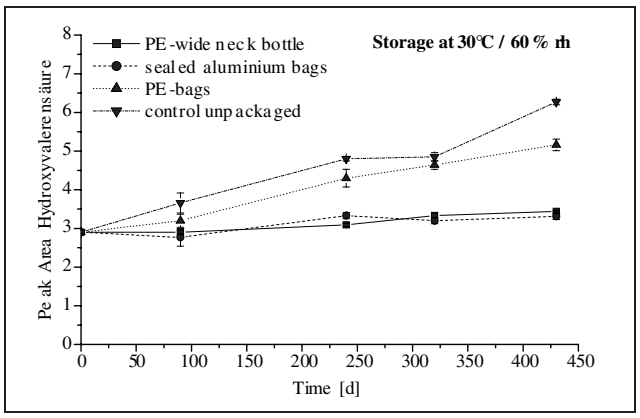

Fig. 14: Increase of hydroxyvalerenic acid in ethanolic valerian extract in different packaging forms at 30 °C/60% rh

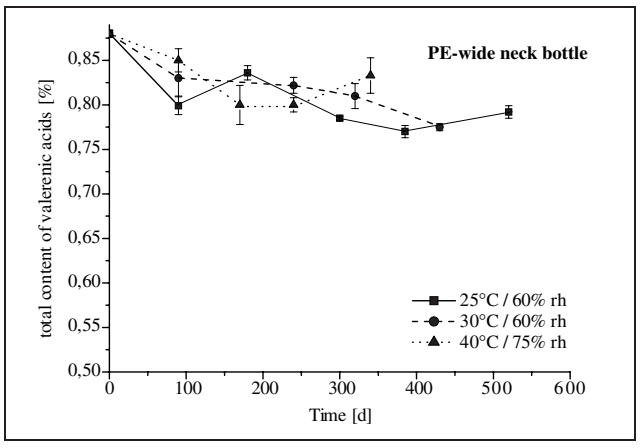

Fig. 17: Changes in total content of valerenic acids of ethanolic valerian extract packaged in wide neck bottles depending on storage conditions

crease of hydroxyvalerenic acid. Storage conditions of 40 °C/75% rh caused a decrease of acetoxyvalerenic acid for the two samples. The overall decrease of acetoxyvalerenic acid of 61%, was correlated to an increase of hydroxyvalerenic acid of 170%.

Although hydroxyvalerenic acid as degradation product was increased, however, no increase of lignan-aglyca could be observed for the plant material.

In addition to the individual valerian acids, also the total content of sesquiterpenic acids and the rate of hydroxyvalerenic acid was analyzed for the unpackaged extract (Fig. 18) and the extract in PE wide neck bottle (Fig. 17). In both cases the content of sesquiterpenic acids decreased by about 14–20%. In conclusion, there was no obvious difference with respect to storage conditions and packaging forms.

Comprehensive and reliable information on differences in stability behaviour can be obtained by comparing the hydroxyvalerenic rate to the content of total sesquiterpenic acids. Whereas no change of proportions can be recog-

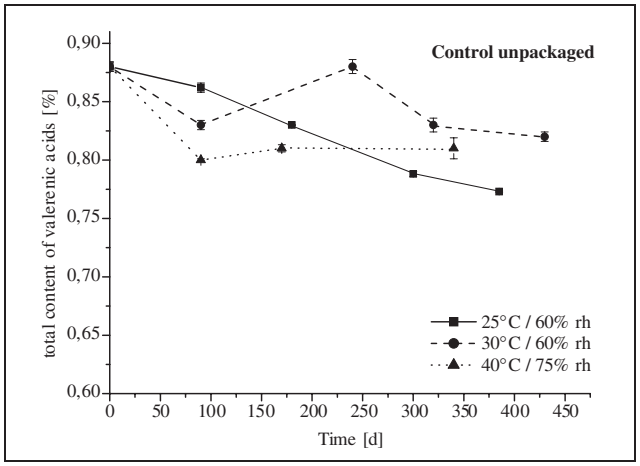

Fig. 18: Changes in total content of valerenic acids of unpackaged ethanolic valerian extract depending on storage conditions

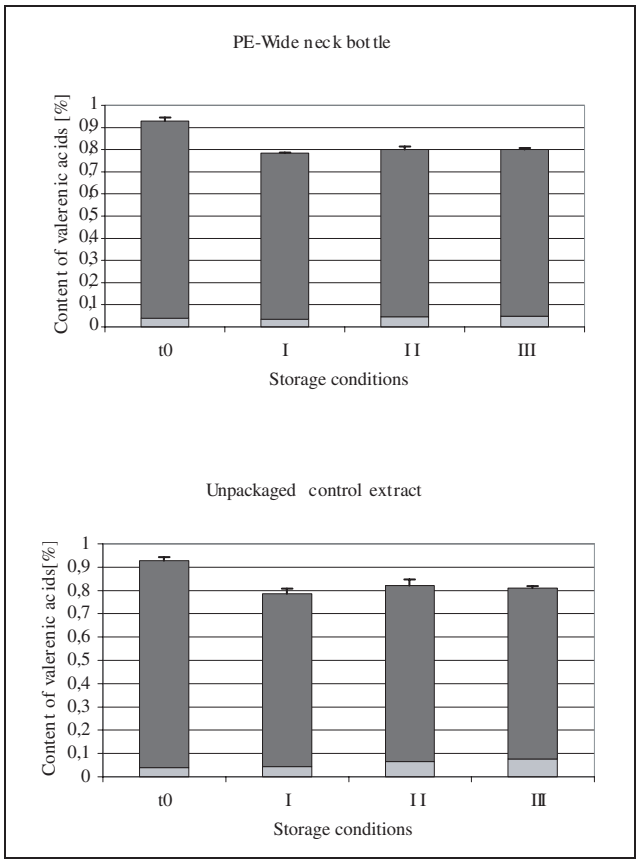

Fig. 19: Comparison of hydroxyvalerenic acid ratio on total valerenic acid content after one year of storage under different climatic conditions in ethanolic valerian extract packaged in wide neck bottles and unpackaged. I: 25 °C/60% rh; II: 30 °C/60% rh; III: 40 °C/75% rh

nized for the extract in PE wide neck bottle, the rate of hydroxyvalerenic acid in unpackaged extract increased under storage conditions of 30 °C/60% rh and 40 °C/75% rh from 4.4% to 7% and 9%, respectively (Fig. 19).

In conclusion, the ethanolic extracts showed to be less susceptible to changes in chemical composition depending on environmental influences than the crude herbal drug. As a consequence, the evaluation of hydroxyvalerenic acid gives more detailed information about the stability status than the total content of valerenic acid.

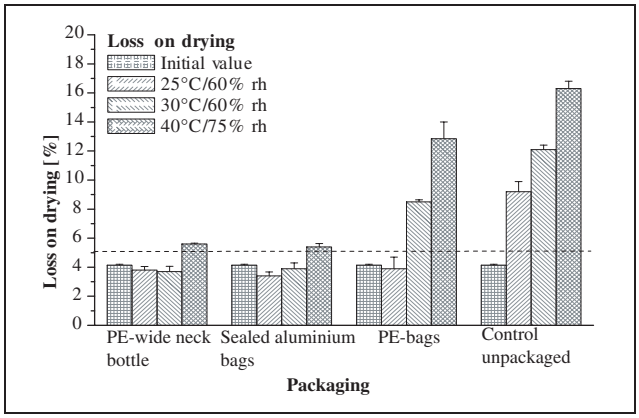

Fig. 20: Loss on drying of ethanolic valerian extracts depending on storage conditions and packaging after three months of storage (dashed line shows allowed upper limit of 5%)

2.4. Physical changes of powdered ethanolic valerian dry extracts

The dry extracts changed their physical properties considerably under the influence of increased moisture. The mesurement of the loss on drying seemed to be a suitable method to elucidate the detected changes. As demonstrated in Fig. 20, no alteration is recognized for extracts at 25 °C/60% in all packaging forms. Extracts in wide neck bottles and sealed aluminium bags remain within the recommended limits (Ph. Eur. 2002b). But even this packaging forms exceed specification limits at 40 °C/75% rh. PE-bags already exceed the limit under the influence of 30 °C/60% rh. The unpackaged extract exceeds the 5%-limit clearly when submitted to any of the ICH guideline's climatic conditions. The increase in water content proved by an increasing loss on drying correlates with changes in optical appearance and viscosity. Extracts with a loss on drying between 5 and 6% are transformed to a sticky mass and are difficult to redissolve. Extracts with loss on drying higher than 8% liquified, but could easily be dissolved.

In conclusion, valerian extracts when stored under defined climatic conditions turned out to be very sensitive against moisture, even in appropriate packaging forms. The documented high amount of water in extracts stored in PE-bags and further unpackaged extracts could explain the instability of the marker compounds.

3. Experimental

3.1. Material

Plant material (Valerianae radix Ch.-B.: 01110077) and ethanolic extract (Extr. Valerianae e rad. sicc. Ch.-B.: 01120382) in different packaging forms was obtained by Fa. Finzelberg, Andernach, Germany.

3.2. Storage conditions

Constant storage conditions of 25 °C/60% rh were created in a climatic chamber with central temperature control. Humidity was generated by ultrasonic moisturisation (Aquastar NT, Honeywell inc.). Conditions of 30 °C/60% rh and 40 °C/75% rh were created inside two desiccators placed each in a drying oven, maintained at the respective temperature. Humidity was created using saturated salt solutions placed at the bottom of the desiccator (magnesium nitrate solution for 60% rh and sodium nitrate solution for 75% rh). Linearity of conditions was documentated by manual measurement of temperature and humidity (Digital thermohygrometer, Oregon scientific).

3.3. Packaging

Powdered drug material and alcoholic extracts were stored in 500 ml PE-wide neck bottles. Besides unpackaged extracts in a 100 ml glass beaker

(100 g), extracts in sealed aluminium bags (each containing 40 g) and PE bags (each containing 20 g) were investigated.

3.4. Sample preparation

Powdered plant material (2 g) was extracted with 40.0 ml methanol 60% (Baker) for 15 min at 30 °C by sonification. The solution was filtered through a 125 mm cellulose filter (Schleicher & Schuell, Germany) into a 100 ml volumetric flask. The extraction flask was washed two times with 5.0 ml methanol 60% and the resulting solutions were filtered as well. The filtrate was added to 100.0 ml.
1.0 g of the ethanolic extract was extracted with 40 ml methanol 60% for 15 min at 30 °C by sonification. The suspension was centrifugated at 4000 r/min for 10 min, and the clear upper phase transferred to a volumetric flask and added to 100.0 ml. The solution was filtered by 0.2 µm silicon syringe filters (Roth, Germany).

3.5. HPLC-system

Pumps Waters 515, Autoinjektor Waters 717 plus, column heater, UV-DAD-Detektor Biotek, Software Biotek Kroma System 2000, column: Eurosphere 100-C18 (5 µm, 250 mm, ID 4.6 mm) Knauer, Germany, pre-column Eurospher 100 C18 (5 µm, 30 mm, ID 4.6 mm) Knauer, Germany. Flowrate: 0.8 ml/min. Eluent A: Water (obtained by a Mili-QFplus, Millipore corp.) ; Eluent B: Acetonitril (HPLC-quality, Baker); Temperature: 30 °C. Injektion volume: 20 µl ; Detektion: 225 nm.
Reference substances: Valerenic acid standard solution was obtained from Fa. Finzelberg, Andernach; Purified pinoresinol prepared as shown under 3.7.

Table 3: HPLC conditions

Time (min)	% B
0	2
8	5
25	15
32	15
42	20
60	40
66	65
82	65
85	10
90	10
92	2
97	2

3.6. TLC

For TLC analysis the separation method of Bodesheim (1996) was modified. Plate: Kieselgel 60 F254 20 × 20 cm; Eluent: 50 ml chloroform p.a. (Merck), 15 ml toluol p.a. (Merck), 30 ml methanol p.a. (Merck) and 5 ml ammonia 25% p.a. (Merck); For the detection anisaldehyde/H_2SO_4-reactive was used; Sample preparation was done by extracting 500 mg plant material with 10.0 ml methanol 70% by sonification. The liwuid was filtered, evaporated to dryness and the residue diluted with 1.0 ml methanol. After centrifugation 10 µl of the upper phase was used for TLC over a length of 10 cm. 10 µl Standard solution: 0.5 mg hydroxyvalerenic acid and 0.5 mg pinoresol solved in 5ml of methanol.

3.7. Isolation of lignans and valerenic acids

Valerian root 350 g, treated at 70 °C for 60 days in a drying oven, was extracted by soxhlet extraction with 3 l n-hexan (Merck). The solution was reduced to half by evaporation under pressure, and extracted 4 × with 250 ml methanol 50% p.a. 2 g of the resulting solubilized fraction was chromatographed by medium pressure on a silica column (80 g) by methanolic gradient elution (30ml/h) and UV-detection. The resulting fractions were analysed and classified by HPLC-DAD. The group classification of compound by UV-DAD was in correlation with the spectral data of Bodesheim (1996) for the lignans, and Godau (1991) for the valerenic acids. For pinoresinol, the spectroscopic data of ^{1}HNMR are in correlation with Nabeta et al. (1991). For pinoresinol-4,4′-di-O-β-di-glucoside, the spectroscopic data of ^{1}HNMR are in correlation with Schumacher et al. (2002). For further information supportive spectral data can be obtained.

3.8. Loss on drying

Values for loss on drying were obtained by a gravimetric method according to Ph.Eur. (2002c).

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