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# Stability testing on typical flavonoid containing herbal drugs

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The aim of the presented work was to examine possible changes in the flavonoid pattern of common flavonoid containing herbal drugs during long term and stress testing storage periods. HPLC fingerprint was used to demonstrate the differences in stability of individual flavonoid components. In addition, the total flavonoid content was determined according to the pharmacopoeial photometrical method. Drug material was stored according to the ICH-guidelines at 25 °C and 60% rh (relative humidity) for long term testing over a 24 months period or at 40 °C and 75% rh under stress conditions for 6 months. Increased temperatures of 80 °C and 100 °C were chosen to elucidate possible instabilities of selected flavonoids. As an overall result, during long term testing, no significant changes in the flavonoid pattern can be detected. However, some flavonoid containing herbal drugs (e.g. birch leaves), showed a decrease of most flavonoids when stored at high temperature by an increase in the respective aglycones. Similar results were obtained during storage at 40 °C/75% rh.

## 1. Introduction

An important prerequisite of drug quality is the guarantee of the stability during shelf life. Stability is defined as the maintenance of the quality until the end of the stated shelf life [1]. Herbal drugs and herbal drug preparations fall under the same official regulations as chemically defined substances concerning quality, efficacy and safety [2]. In most cases no data about the stability of the pharmacological active components or marker substances of herbal drugs or herbal drug preparations are documented in the respective monographs. For this reason, it is indispensable to obtain extensive information about stability of pharmacologically/therapeutically active compounds or defined marker substances. Earlier stability investigations were carried out with pharmaceutically used polysaccharides [3] and essential oils [4]. Flavonoids, a wide spread group of herbal compounds, are known for their diversity of physiological and pharmacological effects [5, 6]. The interest in this group of substances increased in recent years. However, no stability data on this important group of natural compounds is found in the literature.

The use of the global spectrophotometrical methods for the assay of flavonoids of the Ph. Eur. [7] has limited impact, because no information about individual compounds can be obtained and further, the determination of flavonoid C-glycosides is often questioned [8]. HPLC is the actual method of choice to investigate the stability of herbal drug components and their possible degradation products. With respect to typical flavonoid containing herbal drugs, HPLC allows to separate a wide spectrum of flavonoids ranging from polar flavonoids to apolar aglyca. The aim of the presented work was to investigate – by means of the global Ph. Eur. method in comparision to HPLC – if changes in the HPLC-fingerprint occur in the respective drug material, influenced by external factors such as temperature and increased humidity under long term and stress conditions as documented in the ICH-guidelines. In addition, the influence of these parameters on variing particle size of the drug material and further the storage in different packing materials was examined. Basis of the following studies was the "Note for guidance on stability testing of existing active substances and related finished products" [9], where the general demands of stability testing are set. A sufficient stability of the respective drug material over a 24 months period for long term testing (25 °C/60% rh) and over a six months period for accelerated testing (40 °C/75% rh) has to be proven.

## 2. Investigations, results and discussion

### *2.1. Long term testing under moderate climatic conditions (climatic zone II, 25 °C/60%)*

The herbal drug material was stored as a whole (w), in cut (c) (4000–3000 μm) or pulverized (p) (710–180 μm) state in paper bags (pa) or aroma protecting bags (ar) under defined conditions (for details, see Experimental). Flavonoids were determined according to the Ph. Eur. method and by HPLC. Figs. 1–3 show results for three examined herbal drugs Birch leaves [7], Elder flowers [10] and Marigold flowers [11]. A comparision of both analytical methods (total flavonoid content [%] vs. total peak area = sum of all flavonoid peaks in a HPLC chromatogram) is given in Figs. 1, 3, 5.

According to the individual Ph. Eur. monographs, the minimum flavonoid content with a range of −10% and +10% is marked. Figs. 1–6 show a relatively constant level of the total flavonoid complex (peak area) over the storage period. The values of different samples are mostly lying within the

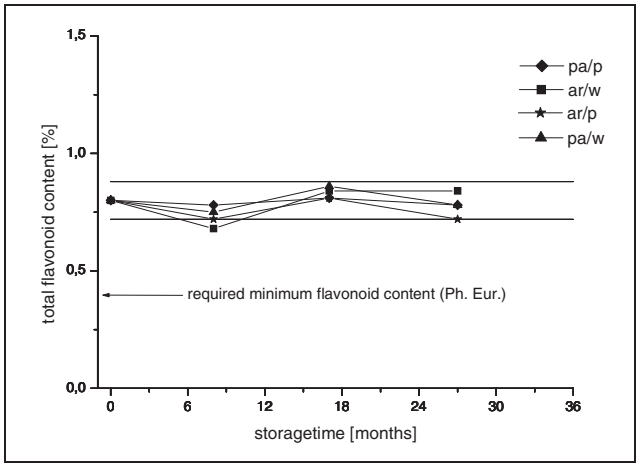


Fig. 1: Flavonoid content of Marigold flowers during long term testing

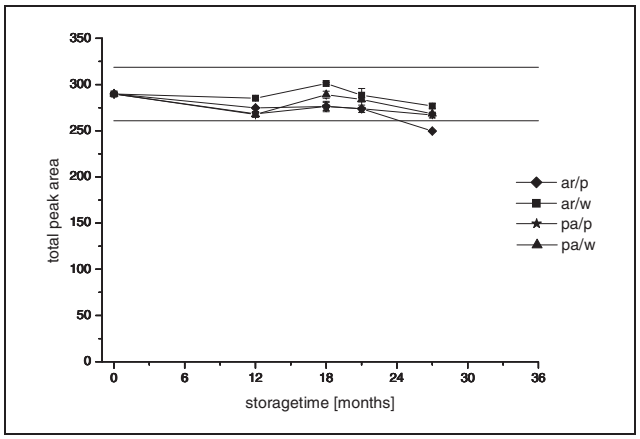


Fig. 2: Total peak area of Marigold flowers during long term testing

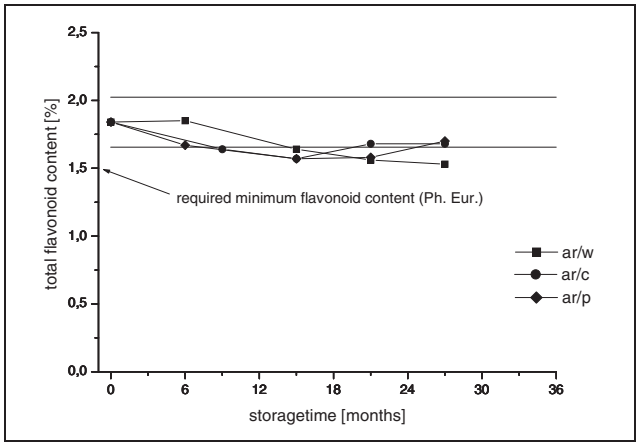


Fig. 3: Flavonoid content of Birch leaves during long term testing

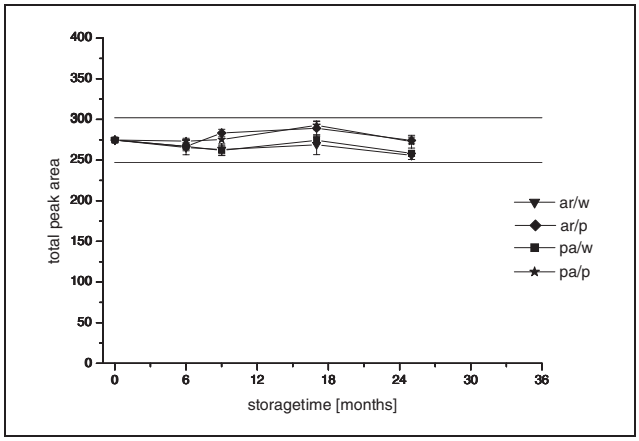


Fig. 4: Total peak area of Birch leaves during long term testing

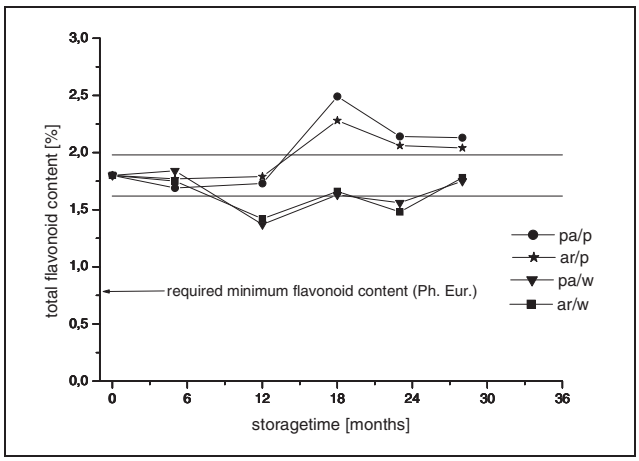


Fig. 5: Flavonoid content of Elder flowers during long term testing

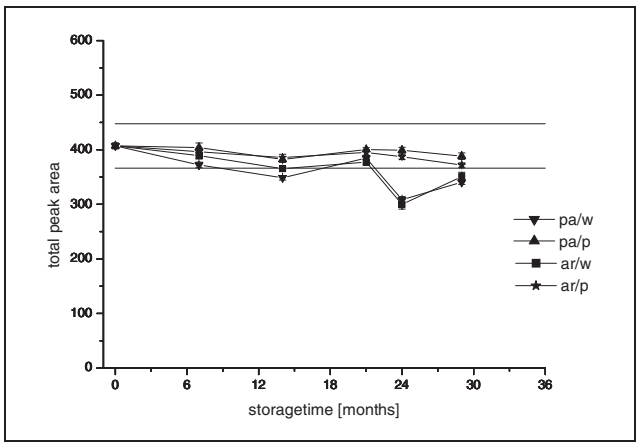


Fig. 6: Total peak area of Elder flowers during long term testing

ranges of −10% and +10% of the initial value. Some values exceed (e.g. see Figs. 3, 5, 6) that interval, which defines the shelf life of pharmaceutical raw materials or final products. It is obvious that the values, obtained by the photometrical method, sometimes show a non linear development (Fig. 5), which may be due to an insufficient reproducibility of the applied analytical method or to problems concerning dissolution of the flavonoids during extraction period (matrix effects). In Fig. 7 the total peak area of flavonoids from Marigold flowers is split in discrete peak areas; the corresponding HPLC chromatogram is shown in Fig. 8. Peaks 1–9 were attributed to flavonoid glycosides by DAD-spectra and by authentic standards, with isorhamnetin-3-rutinoside as leading flavonoid (peak 7). No significant changes of the peak areas 1–9 occurred during the storage period of 27 months. No aglycones as result of a possible degradation could be detected.

These examples represent only a selection of the herbal drugs examined. The data of other flavonoid containing herbal drugs such as Madowsweet flowers, Golden rot herb, Wild pansy herb, Yellow chaste weed flowers and Passion flowers show similar results.

As a consequence of the relative high stability under long term testing conditions, different stress conditions were applied to the herbal drug material, to find out, if flavonoids are stable even at elevated temperatures or increased humidity.

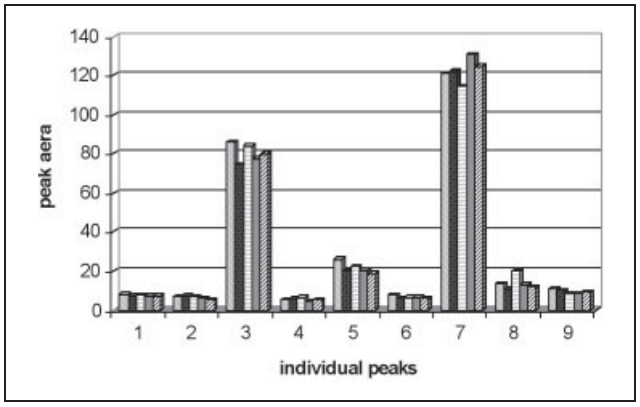


Fig. 7: Variations of flavonoid peak areas of Marigold flowers during storage (25 °C/60% rh) (compare Fig. 8 and 2). ▫ initial value; ■ after 12 months; ▫ after 18 months; ▪ after 21 months; ▨ after 27 month

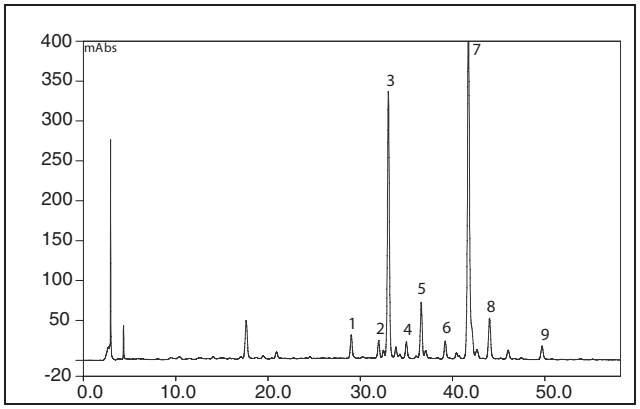


Fig. 8: HPLC-fingerprint of Marigold flower extract. Peak assignment: 1– 4 = not determined; 5 = hyperoside; 6,9 = not determined; 7 = isorhamnetin-3-rutinoside; 8 = isorhamnetin-3-glucoside;

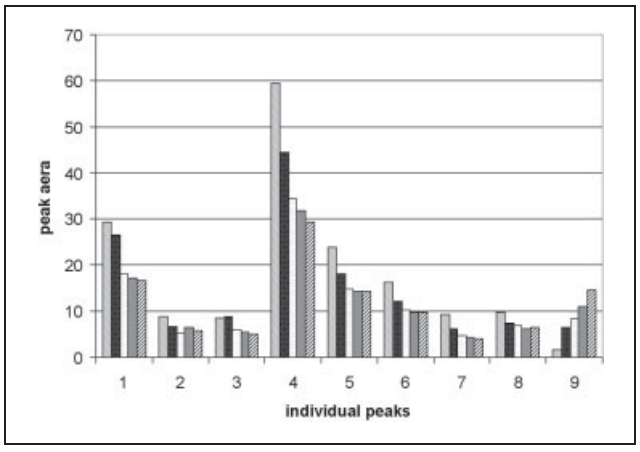


Fig. 9: Variations of flavonoid peak areas of Birch leaves under stress conditions (40 °C/75% rh). ▫ initial value; ■ after 4 weeks; ▫ after 10 weeks; ▪ after 14 weeks; ▨ after 22 weeks. Peak assignment: 1,2 = not detected; 3 = myricitrine; 4 = hyperoside; 5 = isoquercitrine; 6 = avicularine; 7 = not detected; 8 = quercitrine; 9 = quercetine

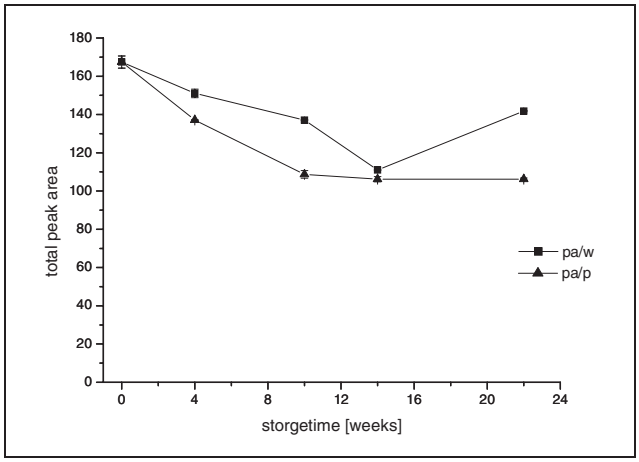


Fig. 10: Total peak area of Birch leaves during accelerated testing (40 °C/ 75% rh)

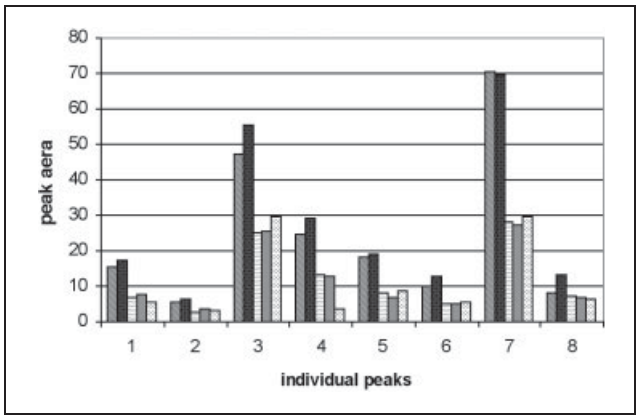


Fig. 11: Variations of flavonoid peak areas of Passion flower under stress conditions (40 °C/75% rh). ▫ initial value; ■ after 4 weeks; ▫ after 10 weeks; ▪ after 14 weeks; ▨ after 22 weeks. Peak assignment: 1,2 = not detected; 3 = shaftoside, isoshaftoside (coelution); 4 = isoorientin; 5 = orientin; 6 = isovitexin-2''-O-glucoside; 7 = vitexine; 8 = isovitexine; 9 = not detected

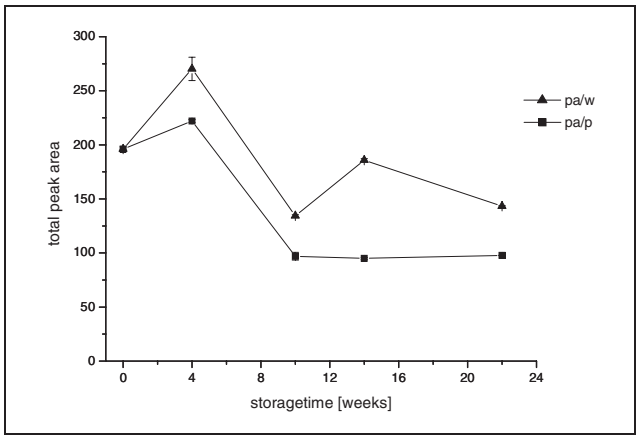


Fig. 12: Total peak area of Passion flower during accelerated testing (40 °C/75% rh)

### *2.2. Stress testing (accelerated testing)*

Storage of Birch leaves and Passion flower at 40 °C and 75% rh for about six months led to a significant decrease in the total flavonoid peak area (Figs. 10, 12). A more rapid drop of the initial value can be noticed during the first three months of storage, an approximately constant level is reached looking at the following test points. It is interesting to note that values for powdered herbal drugs are lower than for the whole drug. This may be caused by a bigger total surface of the herbal drug material and as a consequence a faster degradation. It is further possible that an increase in liberation of the substances during the extraction is taking place. Regarding the individual compounds of the Birch leaves extract (Fig. 9), it be-

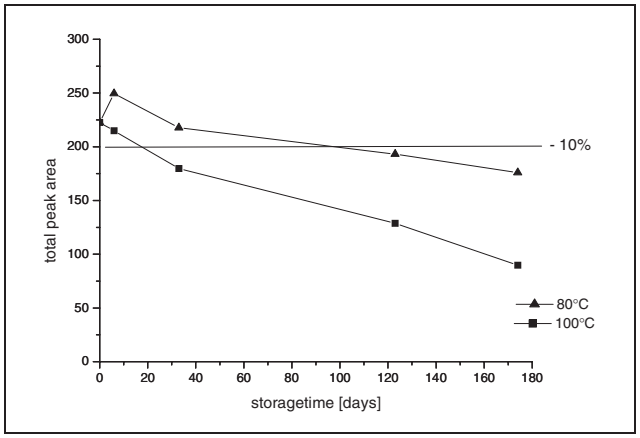


Fig. 13: Peak area of non cut Birch leaves, stored at 80 °C and 100 °C

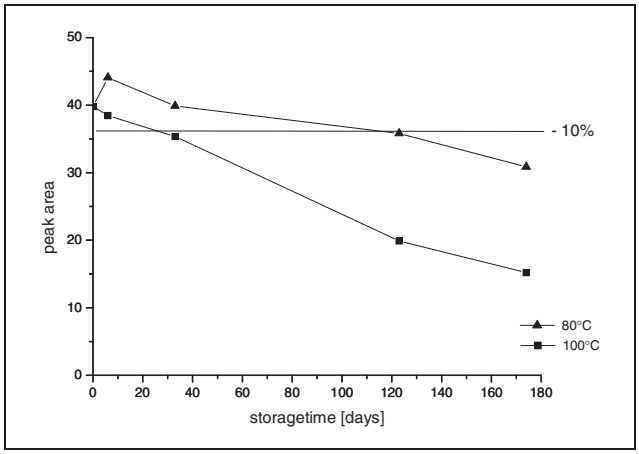


Fig. 15: Peak area of isoquercetine in Birch leaves stored at 80 °C and 100 °C

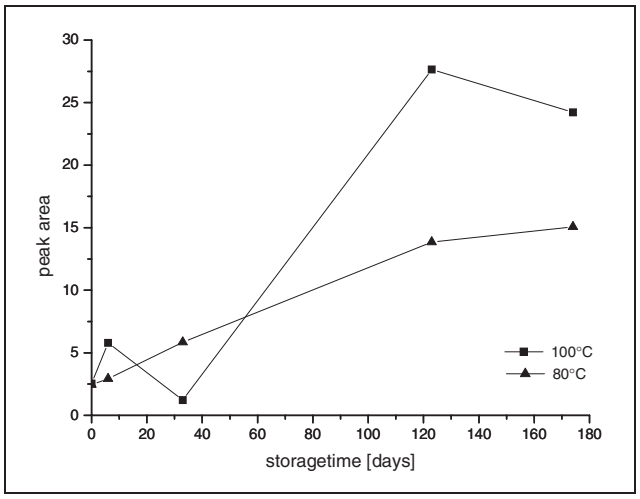


Fig. 14: Peak area of quercetine in Birch leaves stored at 80 °C and 100 °C

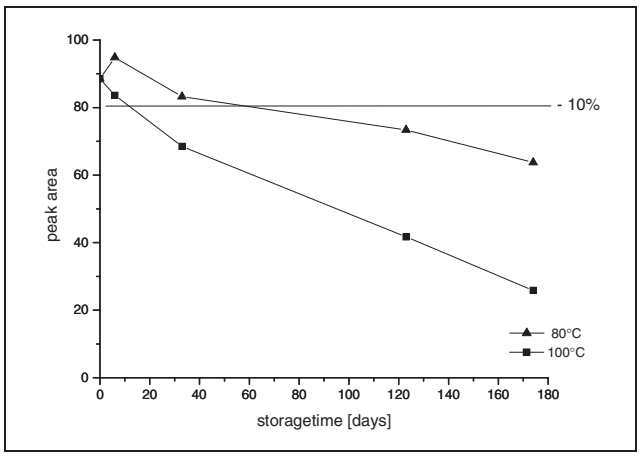


Fig. 16: Peak area of hyperoside in Birch leaves stored at 80 °C and 100 °C

comes obvious that a degradation of genuine flavonoid glycosides into sugar and aglyca-moieties took place. The peak area of most of the quercetine glycosides in Birch leaves show a decrease, the aglycone quercetine increases at the same time. Other degradation products are not detectable. Similar results were obtained for extracts of Passion flower (Fig. 11). Peak areas of nearly all flavonoid glycosides (mostly C-glycosides) decreased, but an expected increase of the corresponding aglycones apigenine/luteoline was not apparent. This shows that even – as one would expect – the more stable C-bonds between sugars and flavonoid moieties are split under these drastic conditions.

In order to examine the stability of flavonoids in the herbal drug material exposed to high temperatures, samples were stored in an oven at 80–100 °C. Such extreme conditions should be useful to elucidate, which individual flavonoid is more susceptible to degradation.

After a 6 months storage a fast decreasing peak area for the sum of flavonoids at 100 °C and a slower decrease at 80 °C in comparision to the initial value (Fig. 13) was obvious. Examples for the stability of individual quercetine-*O*-glycosides are given in Figs. 15 and 16 (isoquercitrine and hyperoside) and for the aglycone quercetine of Birch leaves (Fig. 14). Instabilities for individual flavonoids and the total peak area could be demonstrated. Storage at 100 °C led to a significantly faster decrease than at 80 °C. The marked −10% shelf life level for isoquercitrine (Fig. 15) or the total peak area (Fig. 13) is reached after about three months storage at 80 °C, but already less than one month for a storage at 100 °C. Hyperoside (Fig. 16) shows an even faster decreasing peak area. The peak area of the aglycone quercetine increased to a five to ten times higher value than at the onset of the storage. Nevertheless, it is not possible to make any statement concerning structure related different stabilities of individual flavonoid compounds.

From the presented investigations it can be concluded:

- No significant changes occur in the flavonoid pattern under conditions of long term testing (25 °C/60% rh).
- Under stress conditions (accelerated temperature or humidity), a decrease of the total flavonoid content and an increase of aglycones is obvious. Under usual storage conditions, the stability of flavonoids can be guaranteed at least for a two year period.

## 3. Experimental

### *3.1. Spectrophotometrical determination of flavonoids*

According to the monograph "Birch leaves", No. 1174 Ph. Eur. (2002) [7]

### *3.2. HPLC: Sample preparation*

Powdered plant material (1.0 g) was refluxed with methanol 50% (v/v) for 30 min, methanol was decanted into a 100 ml volumetric flask. The drug material was extracted for further 15 min. By washing the round flask, methanol 50% was added to 100 ml. The extract was filtered through a cellulose filter (125 mm) Schleicher & Schüll, Germany, by removing the first 25 ml. The filtrate was directly used for HPLC-analysis (30 µl injected).

**Table: Employed HPLC-gradient**

| Time (min) | Solvent A (%) | Solvent B (%) | |
|---|---|---|---|
| 0 | 90 | 10 | |
| 0–25 | 87.5 | 12.5 | linear |
| 25–45 | 80 | 20 | linear |
| 45–55 | 74 | 26 | linear |
| 55–65 | 57 | 43 | linear |
| 65–70 | 100 | 0 | linear |
| 70–73 | 100 | 0 | wash |
| 73–80 | 90 | 10 | equilibrate |

### *3.3. HPLC-system and conditions*

Pumps Waters 515, Autoinjector Waters 717 plus, column heater, DAD-detector Biotek 545 V, Software Biotek Kroma System 2000. column: Eurosphere 100-$C_{18}$ (5 µm, 250 mm, ID 4,6 mm) Knauer, Germany. Flow-rate: 1 ml/min. Eluent A: 10% acetonitrile (v/v) in phosphoric acid 5% (v/v), eluent B: 90% acetonitrile in phosphoric acid 5% [12]. Detection wavelength: 340 nm.

### *3.4. Storage conditions*

The design of stability testing was based on the ICH guideline "Note for guidance on stability testing of new drug substances and products" [8]. The guideline imposes constant storage conditions of 25 °C/60% RH (24 months) for long term testing and 40 °C/75% RH (6 months) for accelerated testing, simulating the different climatic zones. Samples were stored in climatic chambers with defined temperature and humidity. Storage at 25 °C and 60% RH was realized in a chamber with central temperature control, humidity was generated by ultrasonic moistener. Conditions of 40 °C and 75% were simulated in an exsiccator with saturated salt solution (sodium nitrate) at the bottom, placed in an drying oven at 40 °C. In addition to the prescribed conditions, stress testing at higher temperatures such as 80 °C and 100 °C for short time is practised, to check possible accelerated degradation of labile compounds. These temperatures were generated for the herbal drug material in a conventional drying oven.

Paper bags (pa) were commercially used package material for herbal teas, Wepa, Amberg, Germany.
Aroma protecting bags (ar) inside covered by layers of aluminium and polyethylene were obtained from Schröder & Wagner, Rinteln, Germany.

Abbreviations: pa: paper bag
ar: aroma protecting bag
w: whole drug
c: cut drug
p: powdered drug

Annotations: Standard deviation is given in Figs. 2, 4, 6, 12 with quadruple determination of values
Lines in the figures indicate a range of ± 10% of the initial value
Total peak area: calculated sum of flavonoid peaks

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