JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

The Glucosinolate−Myrosinase System in Nasturtium (*Tropaeolum majus* L.): Variability of Biochemical Parameters and Screening for Clones Feasible for Pharmaceutical Utilization

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Leaves of *Tropaeolum majus* L. contain high amounts of the glucosinolate glucotropaeolin. They are used in traditional medicine to treat infections of the urinary tract. When *Tropaeolum* leaves are consumed, glucotropaeolin is hydrolyzed to yield mustard oils, which are absorbed in the intestine and excreted in the urine, exhibiting their antimicrobial activity. For a corresponding phytopharmacon, a sufficiently high glucotropaeolin concentration is required and any degradation of glucosinolates while drying must be minimized, i.e. the post mortal cleavage by myrosinases, which are activated by ascorbic acid. In extensive screenings, the dominant parameters determining the glucotropaeolin content in the dried leaves were quantified. It turned out that the glucotropaeolin concentration in the dried leaves represented the most suitable screening parameter. The screening of several hundred *Tropaeolum* plants resulted in the selection of eight high-yield varieties, from which in vitro plants had been generated and propagated as a source for large field trials.

KEYWORDS: Glucosinolates; glucotropaeolin; myrosinase; ascorbic acid; mustard oils; variability; nasturtium; *Tropaeolum majus*

INTRODUCTION

When glucosinolate-containing plants are injured, mustard oils, i.e. isothiocyanates, thiocyanates or related nitrile compounds, are liberated. This post mortal process is triggered by the hydrolytic cleavage of glucosinolates by myrosinases (thioglucoside glucohydrolases, E.C. 3.2.1.147). In general, these enzymes are activated by ascorbic acid (**Figure 1**; for reviews see refs *1*−*3*). Degradation takes place, when tissues of glucosinolate-containing plants are damaged and cells are destroyed, generating a mixing of glucosinolates, myrosinases and ascorbic acid. Glucosinolates and their degradation products, respectively, are important factors in plant defense against herbivores, as well as against pathogens (for reviews see refs *4, 5*). Many glucosinolate-containing plants (e.g., cabbage, kale, broccoli, Brussels sprouts, cauliflower, and horseradish) are used by humans as foods or spices. Indeed, the human metabolism often is impaired by glucosinolates and their degradation products, e.g. due to their goitrogenic effects (*6*). However, the consumption of glucosinolate-containing vegetables apparently has also many positive effects on human health. Besides their potential to reduce the risk of developing cancer significantly (*7, 8*), especially their antimicrobial properties are well-known and used pharmaceutically (*9*). In this context, nasturtium (*Tropaeolum majus* L.), a traditional medicinal plant, is used to treat infections of the urinary tract (*10*). In contrast to most other glucosinolate plants, *T. majus* contains just one type of glucosinolate (*11*), i.e. the benzylglucosinolate, also named glucotropaeolin. When *Tropaeolum* leaves are consumed, due to the disintegration of cells in the course of passing the digestive system, glucotropaeolin is hydrolyzed and benzyl isothiocyanate is formed as a major degradation product. It is absorbed by the intestine and transformed to mercapturic acid derivatives by attaching glutathione (*12*). In the kidneys, these compounds are excreted into the urine, where great amounts are rehydrolyzed to yield again benzyl isothiocyanate, which then develops its antimicrobial activity by inhibiting the growth of bacteria causing the inflammations of the urinary system. However, this conjuncture requires that the amount of glucotropaeolin primarily consumed must be high enough to ensure sufficient amounts of benzyl isothiocyanates in the urine. Thus, when nasturtium products should be used as phytopharmacon in modern medicine, the daily glucotropaeolin uptake must be at least 150 mg to ensure the required agent concentration (*10*). Provided that this dose should be taken up in maximal 10 pills (containing about 300 mg of dried leaf material), a glucotropaeolin concentration of at least 50 mg/g d.w., corresponding to ∼120 μmol/g d.w., is

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10.1021/jf802053n CCC: $40.75
Published on Web 11/06/2008

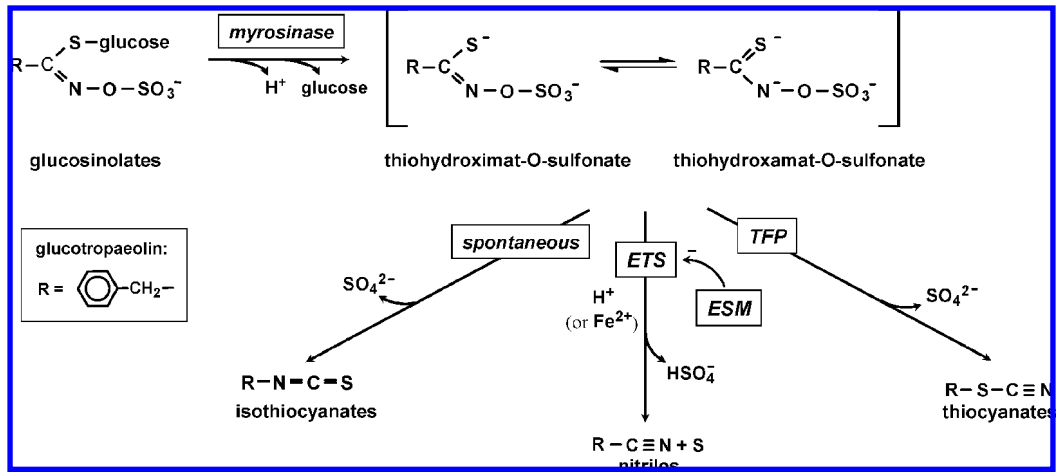

Figure 1. Mustard oil formation. Glucosinolates are degraded by myrosinases. After hydrolytic cleavage, the unstable intermediates rearrange. In general, the main reaction products are isothiocyanates, but, depending on the reaction conditions, nitriles and thiocyanates are also produced. ETS: epithiospecifier protein. ESM: epithiospecifier modifier. TFP: thiocyanate forming protein.

required. Consequently, for pharmaceutical practice, only *Tropaeolum* plants with outstanding high glucotropaeolin content could be used (*13*). An additional problem is related to the fact that varying amounts of glucosinolates might be degraded due to ongoing decompartmentation processes in the course of postharvest processing, especially during drying. Thus, the corresponding liberation of mustard oils and the related loss of glucosinolates, respectively, must be minimized. Consequently, glucotropaeolin-rich *Tropaeolum* plants exhibiting only very little myrosinase activity are required. Unfortunately, neither data on the variability of the glucotropaeolin-content and the myrosinase activity in nasturtium plants are available nor are studies related to the corresponding breeding approaches that have been performed so far. This paper deals with the variability of the biochemical parameters influencing the glucotropaeolin concentration in the dried drug and the related consequences for corresponding screening approaches for nasturtium clones feasible for pharmaceutical utilization.

MATERIALS AND METHODS

Plant Material. All *Tropaeolum majus* (Tropaeolaceae, Brassicales) plants used for this investigation were cultivated from seeds obtained from various horticultural stores and local market-gardens. Additionally, seed material was provided from Dreluso Pharmazeutika (Hessisch-Oldendorf, Germany). Plants were grown in experimental fields with about 70 cm spacing. Two weeks before sowing, the ground was treated with an organic-mineral fertilizer (Compo Guano, Münster, Germany), corresponding to amounts of 55 kg of nitrogen, 30 kg of phosphate and 20 kg of magnesia oxide per hectare.

Sample Preparation. In order to exclude variations in the glucotropaeolin content due to differences in the individual development (*13*), all *Tropaeolum* leaves were harvested from plants exhibiting massive flowering activity, corresponding to a cultivation period of about 12 to 14 weeks after sowing. For the quantification of the glucosinolate content in the vital plants and the myrosinase activity, directly after detaching the leaves—still in the field—the plant material was shock frozen in liquid nitrogen. As the glucotropaeolin content also strongly depends on the developmental stage of the leaves (*13*), only fully expanded, mature and nonsenescent leaves were harvested. In each case, 15 leaves of each plant were pooled as one sample in order to exclude any intraindividual variations. The material was homogenized with mortar and pestle in liquid nitrogen, and subsequently freeze-dried. Aliquots of the lyophilized materials were used for the various determinations. For the quantification of the glucotropaeolin content in the dried plant material, 15 leaves of each plant were dried in a drying oven at 40 °C. Also for the estimation of ascorbic acid, 10 leaves from each plant were frozen in liquid nitrogen, however, freeze-drying was omitted.

Quantification of Glucotropaeolin. Quantitative analyses of glucotropaeolin were performed by HPLC. For this purpose, 40 μL of arbutin (10 mM) was added as internal standard to each 20 mg aliquot. The samples were extracted three times with 1 mL of MeOH (80%, containing 8.5 mM ammonia acetate). Extraction was boosted using ultrasonification (10 min at 50 °C). After centrifugation (15 min at 10000*g*), the supernatants were pooled and concentrated by evaporation to final volumes of about 200 to 300 μL. After the estimation of the exact volumes, water was added to yield exactly 600 μL of aqueous samples. Then 1280 μL of ammonia acetate (43.5 mM) and 120 μL of MeOH were added to obtain a final volume of exactly 2 mL and to achieve the composition of the HPLC eluent A (see below).

HPLC analysis was performed using a RP 18 column (250 × 4 mm). Elution was achieved by applying a one step gradient (8 min eluent A, 3 min eluent B; A, 6% MeOH, 40 mM ammonia acetate; B, 14% MeOH, 40 mM ammonia acetate). For the detection of glucotropaeolin and arbutin, absorbance was recorded at 230 nm. In order to eliminate all undesired substances from the column, a rinse cycle with 80% MeOH (10 min) and a re-equilibration step (25 min, eluent A) were introduced after each chromatography. Based on the peak areas of glucotropaeolin and the internal standard, the amounts of glucotropaeolin were calculated.

Determination of Myrosinase Activity. Myrosinase activity was determined according to Kleinwächter and Selmar (*14*). Using an Ultra Turrax homogenizer, 100 mg of freezed dried plant powder was extracted with 10 mL of 25 mM phosphate buffer (pH 5.7). The homogenate was filtered through cheese cloth followed by a centrifugation (30000*g* for 1 h). To remove the low molecular compounds (i.e., endogenous glucotropaeolin, ascorbic acid, and glucose) 2 mL of the supernatant was applied to a PD-10 desalting column (GE-healthcare Biosciences). After addition of 0.5 mL of buffer, the enzyme sample was eluted with 2.5 mL of phosphate buffer. The resulting sample was used directly for the myrosinase assay. Ten microliters of enzyme sample was incubated with glucotropaeolin (final concentration, 1 mM; final volume, 1 mL) and ascorbic acid (standard concentration, 2 mM) in a phosphate buffer (25 mM, pH 5.7) at 30 °C. After incubation (20 min), the incubation mixture was shock frozen in liquid nitrogen. 500 nmol of arbutin (i.e., 500 μL of 1 mM) was added as internal standard, and the mixture was freeze-dried. The lyophilized powder was extracted with 1 mL of methanol, followed by a centrifugation (10000*g* for 10 min). The supernatant was transferred in a test tube. The residue was extracted with another portion of methanol (0.5 mL) and centrifuged again. The supernatants were combined and the methanol was evaporated using a stream of air. The residue was dissolved in 200 μL of pyridine and then used for the derivatization and subsequent HPLC-

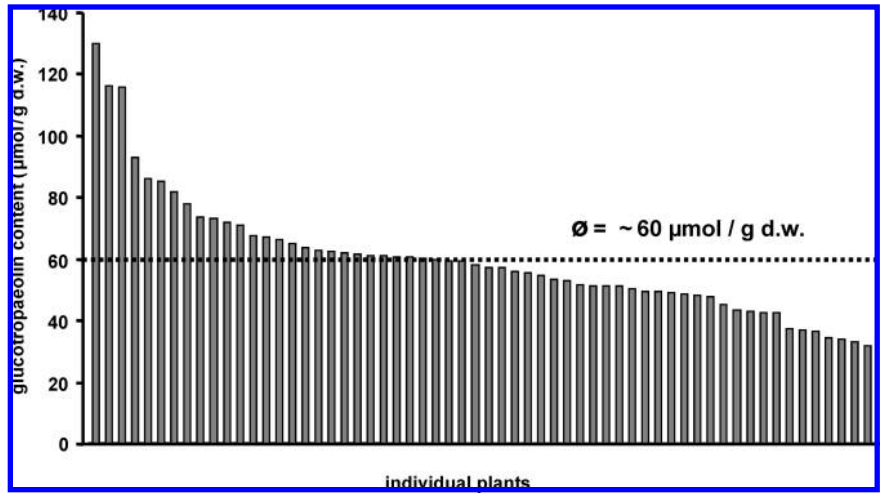

Figure 2. Glucotropaeolin content of *Tropaeolum majus* leaves. In order to avoid any degradation of glucosinolates, leaves had been shock-frozen still in the field. After freeze-drying, glucotropaeolin content was determined by HPLC. All plants derived from commercially available seeds, cultivated under similar conditions. All values are based on at least two independent estimations, bearing maximal methodological variations of less than 1.5%, average value was about 1%.

analysis as described by Kleinwächter and Selmar (*14*). Linearity of reaction velocity during the course of the enzyme assay was ascertained by corresponding incubation series.

Extraction and Quantification of Ascorbic Acid. For the quantification of the ascorbic acid content a commercially available ascorbic acid test, developed for the investigation of foodstuffs, was used (L-ascorbic acid, colorimetric method, Boehringer Mannheim, Germany). This test is based on the reduction capacity of ascorbic acid, which reduces the tetrazolium salt MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazoliumbromide) in the presence of the electron carrier PMS (5-methyl-phenaziniummethosulfate) at pH 3.5 to yield a colored formazane. The concentration of this dye is determined photometrically at 578 nm. While the total amount of reducing agents is quantified in one assay, in a parallel assay, ascorbic acid is oxidized by ascorbate oxidase to yield dehydroascorbate, which will not produce the colored formazane. The actual ascorbic acid concentration will be calculated on the basis of the differences of the absorbance of both assays.

For each analysis, 60 mg of frozen leaf material was homogenized in 750 μL of 15% (w/v) meta-phosphoric acid (containing 4 mM Na_2EDTA, adjusted to pH 3.5) for 3 min using an Ultra-Turrax homogenizer. After centrifugation (10 min at 10000*g*) the supernatant was diluted 1:10 with aqua dest. The entire procedure was performed in an ice bath in the dark in order to prevent degradation of the labile ascorbic acid. Incubation was carried out according to the data sheet 409677 (Boehringer Mannheim, Germany); however instead of 100 μL of diluted sample, 1 mL was used.

RESULTS AND DISCUSSION

Variability of the Glucotropaeolin Contents. In order to determine the natural variability of the glucotropaeolin contents in nasturtium, more than 200 individual plants had been screened in the years 2003 to 2005. However, due to massive annual differences of the weather conditions, which significantly influence the glucosinolate system, the absolute levels of the factors analyzed (glucosinolate contents, myrosinase activity etc.) differ significantly. Thus, in this study only the data for plants that had been grown in one single year (2004) are presented. All plants derived from commercial batches of *Tropaeolum* seeds and had been analyzed over a period of several years. The screening revealed that the glucosinolate content in the leaves varied markedly, in 2004, the values ranged from less than 40 μmol/g d.w. (~16 mg /g d.w.) to more than 130 μmol/g d.w. (~50 mg/g d.w.); the mean value of the 60 individual plants tested in 2004 was about 60 μmol/g d.w. (**Figure 2**). As mentioned above, it should be emphasized that the glucotropaeolin content is also affected strongly by the weather conditions: although in all cases identical seed material was used, and the cultivation conditions were similar, too, the corresponding glucotropaeolin quantifications in three successive years yielded in the quite different mean values of 48, 60 and 78 μmol/g d.w., respectively. This marked influence of the weather and cultivation conditions are subject of another study and will not be considered in detail in this paper, which focuses on the endogenous plasticity of the glucosinolate system in nasturtium. This tremendous variability firmly justifies our approach to screen and select for glucotropaeolin-rich *Tropaeolum* varieties exploitable for pharmaceutical usage. In a corresponding study on the glucosinolate content and myrosinase activity in horseradish (*Armoracia rusticana*), Li and Kushad (*15*) reported also extremely high variation in the levels of these parameters for different genetic varieties. In the same manner, similar high variations are reported for the glucosinolate levels in various lines of *Brassica napus* (*16*). Although the entire regulation of synthesis and accumulation of glucosinolates still is not fully understand, many different factors are thought to be involved in its regulation, e.g. plant development (*17*), temperature (*18*), pathogen attack (*19*), soil−water potential (*20*). Consequently, apart from the variability which directly is due to clonal variations, there might be numerous indirect clonal effects, which influence the glucosinolate level by differing impacts on the various regulative parameters, or their expression, respectively.

Variability of the Ascorbic Acid Contents. The desired high glucosinolate content in selected nasturtium varieties only will gain high glucotropaeolin concentration in the dried leaves, when its degradation in the course of harvesting and drying can be circumvented efficiently. An effective restriction of the undesired mustard oil liberation could be implemented by the abolishment of the corresponding hydrolysis by myrosinases. These enzymes are known to be activated effectively by ascorbic acid (e.g. refs *21, 22*). In contrast to most other myrosinases, which are characterized by a constitutive basal activity even in absence of activating ascorbic acid (*23, 24*) the myrosinase from *Tropaeolum* does not reveal any activity when this activator is missing (*14*). Maximal activity occurs when the ascorbic acid concentration is about 2 mM, corresponding to approximately 2 mmol/kg f.w., concentrations below this level would yield in restricted myrosinase activity. Consequently, one approach was

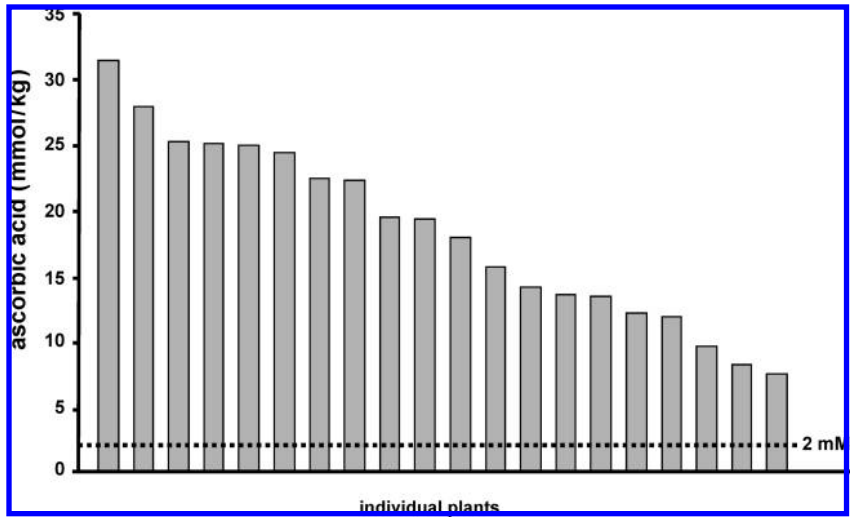

Figure 3. Ascorbic acid concentration of *Tropaeolum majus* leaves. In order to avoid any degradation of ascorbic acid, leaves had been shock-frozen still in the field, and frozen plant material had been transferred directly into meta-phosphoric acid. All plants derived from commercially available seeds, cultivated under similar conditions. All values are based on at least two independent estimations, bearing maximal methodological variations of less than 2.0%, average value was about 1.3%.

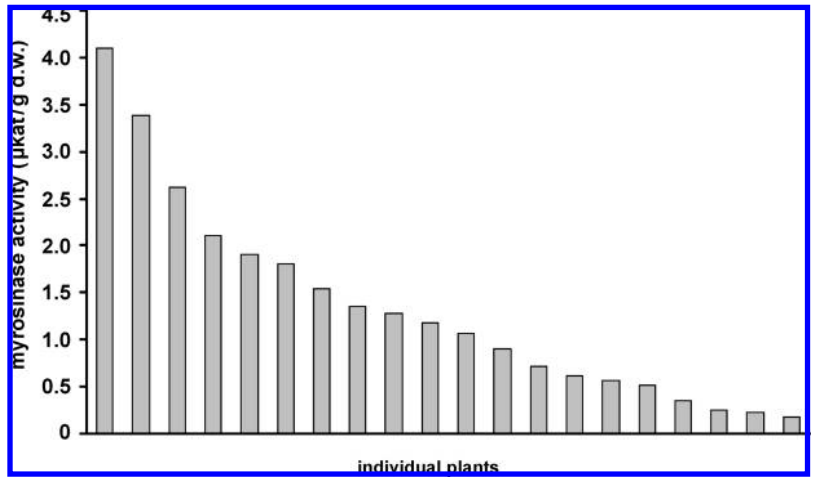

Figure 4. Myrosinase activity in *Tropaeolum majus* leaves. Leaves had been shock-frozen still in the field. After freeze-drying, myrosinase was extracted with a phosphate buffer. Enzyme activity was determined using an assay containing 2 mM ascorbic acid to ensure complete activation, and 1 mM glucotropaeolin as substrate. All plants derived from commercially available seeds, cultivated under similar conditions. All values are based on at least two independent estimations, bearing maximal methodological variations of about 10%.

aimed to screen for nasturtium plants with very low concentration of ascorbic acid.

Similar to the high variations in glucotropaeolin content, also the concentration of ascorbic acid varies tremendously, ranging from nearly 35 mmol/kg f.w. to less than 10 mmol/kg f.w. (**Figure 3**). However, even the lowest concentration of ascorbic acid determined was still 4 times higher than the ascorbic acid concentration necessary for maximal activation of myrosinase. Thus, although the ascorbic acid concentration varies enormously, it is not a suitable parameter for the screening of *Tropaeolum* varieties exploitable for pharmaceutical usage.

Variability of the Myrosinase Activity and Loss of Glucosinolates while Drying. The data mentioned above evinces that in all *Tropaeolum* plants analyzed so far the myrosinase is activated completely when the leaf tissue is disrupted, e.g. in the course of disintegration due to harvest and drying. Consequently, further screening was focused on the actual myrosinase activity, fully activated by ascorbic acid. As pointed out above, the range of variation of both parameters, the glucotropaeolin content and the ascorbic acid concentration, was markedly high, corresponding to 5-fold differences between highest and lowest values. In contrast, the myrosinase activity even varied in a far higher range from less than 0.2 μkat/g d.w. to more than 4 μkat/g d.w., corresponding to a 25-fold difference (**Figure 4**). The question arose, if the relatively low myrosinase activity in various nasturtium plants indeed hampers or at least limits the post mortal hydrolysis of glucosinolates or if such low activities might already be sufficient for effective mustard oil liberation. A reliable approach to investigate these coherences is the analysis of the actual losses in glucosinolate content and their comparison with the corresponding myrosinase activity.

The potential loss of glucosinolates during drying was estimated by comparing the actual glucotropaeolin concentrations in fresh leaves with those of the dried material from the same individual plant. As a comparison of various drying techniques revealed that the loss of glucosinolates in *Tropaeolum* leaves is minimal when the fresh nasturtium leaves are dried in a laboratory oven at 40 °C (*25*), this procedure was applied for the current investigation. As predicted, significant amounts of glucotropaeolin had been degraded while drying, yet the actual losses varied strongly (**Figure 5**). In some cases, only traces of

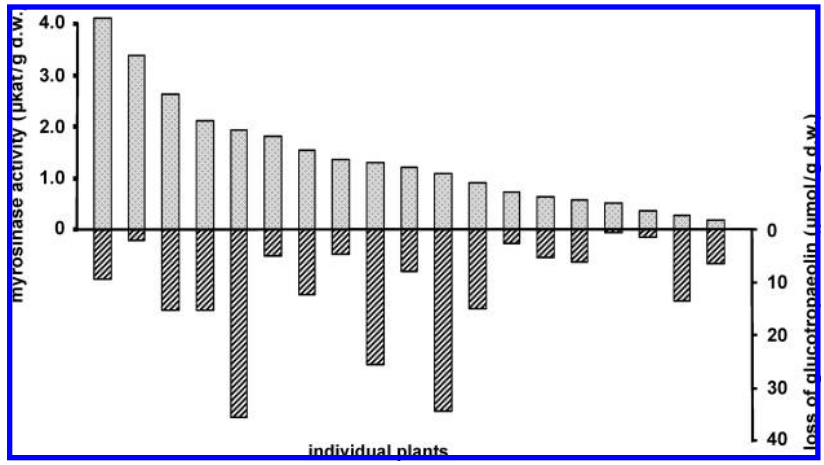

Figure 5. Comparison of myrosinase activity and loss of glucotropaeolin while drying of *Tropaeolum majus* leaves. Myrosinase activity and the loss of glucotropaeolin are displayed for each individual sample. The corresponding losses of glucosinolates were calculated by the differences of glucotropaeolin contents in fresh and dried leaves of each individual plant.

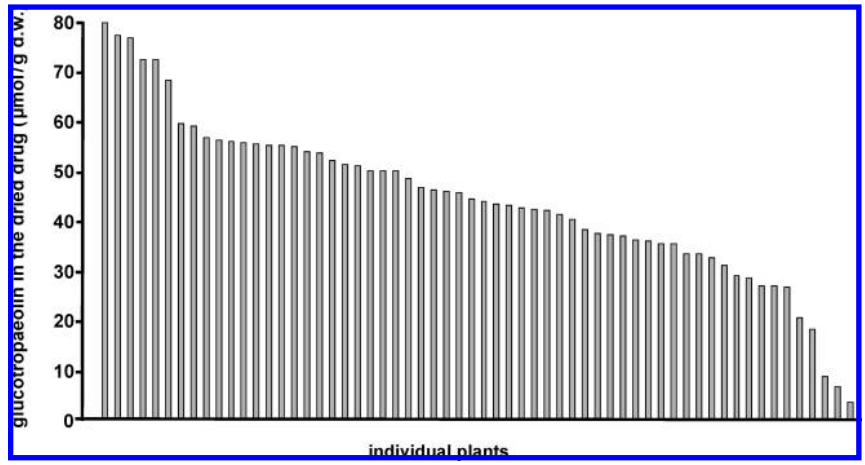

Figure 6. Glucotropaeolin contents in dried leaves of 60 individual *Tropaeolum majus* plants. After oven-drying at 40 °C, the glucotropaeolin content was determined by HPLC. All samples derived from plants, grown from commercially available seeds, and which had been cultivated under similar conditions. All values are based on at least two independent estimations, bearing maximal methodological variations of less than 2.5%, average value was about 1.4%.

glucosinolates had been hydrolyzed, whereas in others, more than 35 μmol/g d.w. disappeared, corresponding to a deficit up to 45%.

Astonishingly, the strong differences in glucosinolate degradation are not correlated with the actual myrosinase activity, and minor activities detected in certain nasturtium plants seem to be not limiting the glucotropaeolin hydrolysis induced by drying, e.g. in plants bearing only about 0.3 μkat/g d.w., more than 15 μmol/g d.w. are lost. Obviously, other factors than the actual content of myrosinase activity are determining the extent of glucosinolate degradation while drying. It can be assumed that especially the stability of the cellular structures is important. In this context, the thickness of the cell walls is of special interest. The thicker the cell walls are, the lower is the probability that the cells are collapsing when the turgor decreases and that due to the ongoing decompartmentation glucosinolates are hydrolyzed. Moreover, it cannot be excluded that other factors, which are induced in the course of drying, may play an important role, i.e. MBPs, proteins which bind to myrosinases (*26*) or MyAPs, which are associated with these enzymes (*27*). In order to investigate putative interrelations between the glucosinolate content, the ascorbic acid concentration and the myrosinase activity, correlation analyses had been performed. In all cases, the correlation factor (r) was between 0.1 and 0.2, demonstrating that there is any correlation between the levels of the three parameters analyzed.

Variability of Glucosinolate Contents in the Dried Leaves. As any correlation between the actual myrosinase activity and the loss of glucotropaeolin could be detected, as basis for reliable screenings for *Tropaeolum* varieties exploitable for pharmaceutical usage—apart from the glucotropaeolin concentration in the fresh leaves—only the actual losses of glucosinolates during drying remain as a significant screening parameter. Both factors are combined by quantifying the content of glucotropaeolin in the dried leaves. Consequently, the screening was focused on this property. As predicted, also this parameter showed very broad variations. The corresponding quantification of oven-dried leaves of 60 individual plants yielded glucotropaeolin concentrations ranging from less than 5 μmol/g d.w. to more than 80 μmol/g d.w. (**Figure 6**). In consequence, a cultivation of *Tropaeolum* plants generated from undefined and unselected seed material could not be recommended for bulk production. In this case—due to the presence of plants revealing only a very low glucotropaeolin content—the overall drug quality decreases. This should be avoided by cultivating special high-yield varieties, selected out of the gene pool. An appropriate method for mass propagation of selected varieties could be achieved

by in vitro propagation techniques. To ensure that the desired genetic homogeneity is not abolished by somaclonal variations, propagation should be based on multiple sprout formation techniques.

In this manner, from the *Tropaeolum* plants raising the highest glucotropaeolin concentration in the dried leaves in vitro plants had been generated. Finally, 8 corresponding clones were propagated by multiple sprout formation techniques. In total 10,000 of the produced plants will be cultivated in a field trial (Thiele, 2008), in order to provide a solid basis for the farming of *Tropaeolum majus* for pharmaceutical usage. In this context, it should be noted that—in addition to the selection of the high-yield varieties—the optimization of cultivation conditions is also required. In doing so, special focus had to be put on the application of sulfur, by which the glucotropaeolin content in nasturtium could be enhanced (*28*).

ACKNOWLEDGMENT

The authors wish to thank T. Köhler (Deluso Pharmazeutika, Hessisch Oldendorf) for providing seed material of *Tropaeolum majus* and technical support.

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Received for review July 7, 2008. Revised manuscript received September 11, 2008. Accepted September 30, 2008. This study was supported by the Fachagentur für Nachwachsende Rohstoffe e.V. (FKZ: 22018101).

JF802053N