

# Concentration and Composition of Flavonol Glycosides and Phenolic Acids in Aerial Parts of Stinging Nettle (*Urtica dioica* L.) are Affected by Nitrogen Fertilization and by Harvest Time

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## Summary

Plants of *Urtica dioica* were cultivated in three years with four different nitrogen (N) levels (0, 100, 200 and 400 kg N ha<sup>-1</sup>) and the aerial parts were harvested three times per year to investigate if the content of flavonoids and phenolic acids were affected. The flavonol glycosides quercetin-3-*O*-glucoside, quercetin-3-*O*-rutinoside, isorhamnetin-3-*O*-rutinoside, and kaempferol-3-*O*-rutinoside and the phenolic acids 5-*O*-caffeoylquinic acid, 3-*O*-caffeoylquinic acid, 5-*O*-feruloylquinic acid and 2-*O*-caffeoylmalic acid were identified and quantified in extracts of the aerial parts of *U. dioica* by LC-DAD-MS and RP-HPLC-DAD, respectively. High N-levels reduced the concentration of total flavonoids significantly from an average of 10 mg g<sup>-1</sup> dry matter

(DM) at 0 kg N ha<sup>-1</sup> to 5 mg g<sup>-1</sup> at 400 kg N. The effect of N levels on total phenolic acids was only significant in the second harvest each year with a reduction from an average of 30 mg g<sup>-1</sup> DM at 0 kg N ha<sup>-1</sup> to 23 mg g<sup>-1</sup> at 400 kg N ha<sup>-1</sup>. The composition of flavonoids changed significantly among the major flavonoids with later harvest time resulting in an increase in the content of quercetin-3-*O*-rutinoside and a decrease of the quercetin-3-*O*-glucoside content. From the present study it appears that cultivation of *U. dioica* herba for medicinal purposes with a high yield of bioactive compounds is a compromise between a high yield of plant material and the content of flavonol glycosides and phenolic acids in the harvested product.

**Key words.** cultivation – medicinal plant – phenolics – caffeic acid derivatives – isoquercitrin – rutin

## Introduction

Stinging nettle (*Urtica dioica* L.) is used as a herbal remedy for the treatment of many diseases. The aerial parts of the plants are used as a blood purifier and diuretic, and infusion of the plant is used for the treatment of nasal and menstrual haemorrhage, diabetes, rheumatism, eczema, and against diarrhoea as well as in the treatment of arterial hypertension (WAGNER et al. 1989; ZIYYAT et al. 1997). Previous studies on water extracts of the aerial parts of *U. dioica* have in addition to a diuretic and a natriuretic effect also demonstrated a hypotensive action as well as an anti-hyperglycemic activity in rats *in vivo* (TAHRI et al. 2000; LEGSSYER et al. 2002; BNOUHAM et al. 2003) confirming the traditional use of this plant against the development of cardiovascular diseases and diabetes, respectively. Other pharmacological effects, such as stimulation of human lymphocyte proliferation (WAGNER et al. 1989), anti-inflammatory activity (RIEHMANN et al. 1999; AKBAY et al. 2003), anti-ulcer and analgesic activities (GÜLCIN et al. 2004) have been demonstrated for *U. dioica* and further it has been suggested that the immunostimulatory activity of leaf extracts could be the reason for the use of this plant in traditional anticancer treatment (OBERTREIS et al. 1996). The pharmacological effects observed for the aerial parts of *U. dioica* are believed to be due to the content of fla-

vonol glycosides such as quercetin-3-*O*-rutinoside (rutin) and quercetin-3-*O*-glucoside (isoquercitrin), kaempferol-3-*O*-rutinoside and isorhamnetin-3-*O*-glucoside and phenolic acids such as 5-*O*-caffeoylquinic acid (chlorogenic acid) and 2-*O*-caffeoylmalic acid (OBERTREIS et al. 1996; AKBAY et al. 2003; GÜLCIN et al. 2004) (Fig. 1). The content of these phenolics is therefore important for the quality of the plant material sold for the preparation of various medicinal products. This is also in accordance with the quality criteria for *U. dioica* herba given in the pharmacopoeias such as the EUROPEAN PHARMACOPOEIA (2007) where minimum concentrations of bioactive components are listed, including also some of the phenolics mentioned above.

The concentrations of phenolics in plants have been shown to increase in response to biotic and abiotic stress. Different types of plant stress have been investigated in relation to the accumulation of phenolics in plants, including UV-light, drought, plant diseases, insect attack, physical scorching and nutrient deficiency such as low nitrogen availability (BONGUE-BARTELSMAN and PHILLIPS 1995; DIXON and PAIVA 1995; STEWART et al. 2001; AWAD and DE JAGER 2002; NØRBÆK et al. 2003; ANTTONEN et al. 2006). Low fertilizer levels in cultivation resulted in higher concentrations of phenolics in leaves of tomato (BONGUE-BARTELSMAN and PHILLIPS 1995), in apple skin (AWAD and DE JAGER 2002), in leaves

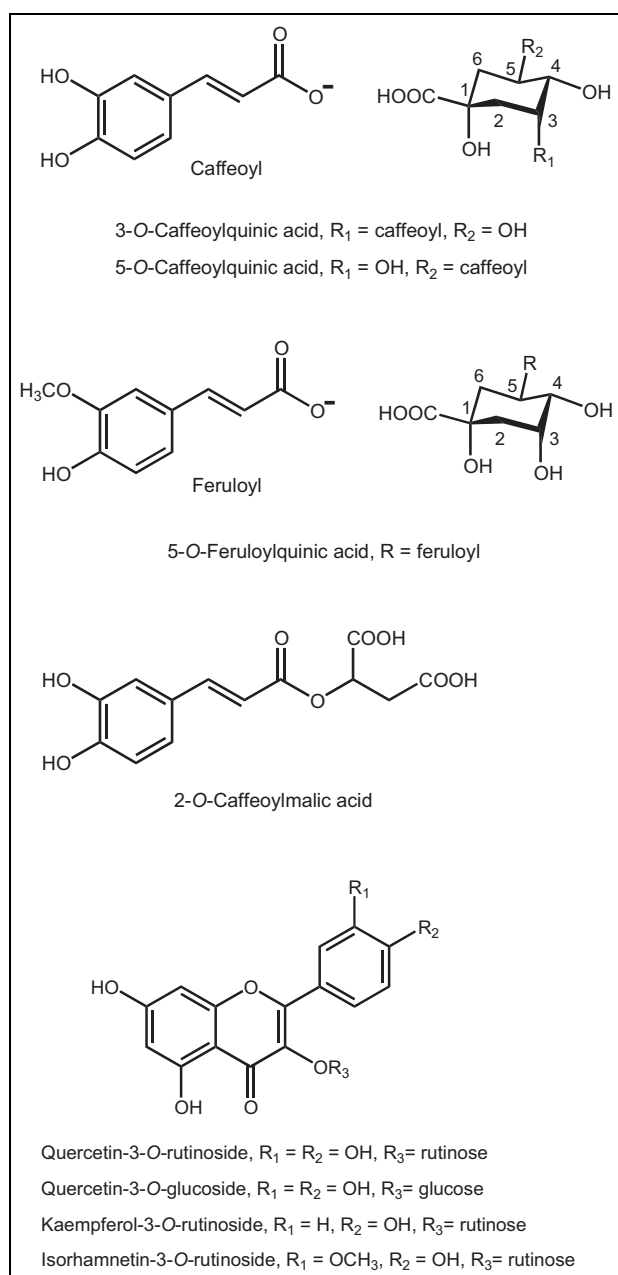


Fig. 1. Flavonoids and phenolic acids identified in aerial parts of stinging nettle (*Urtica dioica*) in the present investigation.

of barley (NØRBÆK et al. 2003) and in fruits of strawberry (ANTTONEN et al. 2006), but no investigations of this fertilizer effect have been made in *U. dioica*. *Urtica dioica* is on the other hand a nitrophile plant and higher amount of nitrogen will therefore produce more plant material to harvest (WEISS 1993). Therefore, we reasoned that increasing the supply of nitrogen fertilizer in cultivation of *U. dioica* will produce more plant material but may alter the concentration and composition of flavonoids and phenolic acids in aerial parts, and hence the quality of the harvested plant material. To test this hypothesis, we used four increasing nitrogen supplies and sampled the aerial parts several times during three growing seasons for analyses of yield, concentration and composition of flavonoids and phenolic acids.

## Materials and Methods

### Plant material

Transplants of stinging nettles for the field experiments were made from root cuttings in April 2003. The plant material for cuttings was obtained from a herb grower (Søren Kristoffersen, SciCom, Vejle, Denmark), and the origin of seeds for this planting was again from a collector of wild herb seeds (Flemming Tommerup, CompleMedic, Denmark) who harvested the seeds in a natural occurring nettle population (Søager near Snoldelev, Sealand, Denmark). Root cuttings of approx. 3 cm in length were placed in speedling plugs (VP 77, Vefi A/S, Larvik, Norway) filled with a soil/sphagnum mixture enriched with organic fertilizer and lime (Pindstrup 301/024 and 327/024 organic transplant mixture, Pindstrup mosebrug A/S, Denmark). The transplants (3000 plugs) were raised in a glasshouse at a minimum temperature of 12 °C and reached a size of 6 to 8 leaves after five weeks.

### Chemicals

Methanol (MeOH), acetonitrile (MeCN) and formic acid were of 99.9 % high-performance liquid chromatography (HPLC) grade (Aldrich-Chemie, Germany). The water used was ultra pure generated by an Elgastat water purification system (Elga Ltd., United Kingdom). All eluents for HPLC were filtered through nylon 0.45 µm Cameo 25P syringe filters (Bie & Berntsen, Denmark) and degassed with ultrasound for 20 min before use. Authentic samples of 5-O-caffeoylquinic acid, quercetin-3-O-rutinoside, quercetin-3-O-glucoside, isorhamnetin-3-O-rutinoside, kaempferol-3-O-rutinoside were purchased (Extrasynthese, France) and 3-O-caffeoylquinic acid was kindly donated by Anne S. Meyer, Department of Chemical Engineering, Technical University of Denmark.

### Field experiments

The field experiment was planted on May 22, 2003 on a sandy loam soil at Research Centre Aarslev (10 °27' E, 55 °18' N). The plants were established in a bed system and through black plastic ground cover to control weeds. The individual plots were 5 m long and 1.5 m wide beds. The plant density in beds was about 16 plants per m<sup>2</sup> in four rows and with a row distance of 0.2 m and an in-row spacing of 0.25 m. The plastic cover was 1.2 m in width and secured with soil along the edges. The spacing between individual plots was 0.80 m. The relative wide spacing between plots was to avoid contamination with nitrogen (N) between neighbouring plots. In the space between plots a lawn grass mixture was sown and kept short by moving all through the three seasons of the experiment. The statistical design of the field experiment was in randomised blocks with three replicates of the fertilizer treatment in 2003 and two replicates in 2004 and 2005.

### Fertilization

The N treatments in the experiment were as follows: 2003: 0, 75, 150 and 300 kg N ha<sup>-1</sup>; 2004: 0, 100, 200 and 400 kg N ha<sup>-1</sup> and 2005: 0, 100, 200 and 400 kg N ha<sup>-1</sup>. The N was applied as urea fertilizer and in 2003 and 2004 all the N was given at the start of the season in

April. In 2005 the fertilizer applied was also urea but given as a split application with 1/3 at the start of the season and the last 2/3 after the first and second harvest (June and August).

#### Harvest time (Re-harvest)

The aerial parts of the nettle plots were harvested at two times in 2003 (July and September) three times in 2004 (May, July and October) and also three times in 2005 (June, August and October). The two first cuts in each season were timed to be at onset of flowering, whereas the last cut in each year were timed after the highest possible yield of fresh material before winter deterioration. The plants were cut about 15 cm over ground and the yield of fresh material was recorded by weighing just after cutting. The plots-area for yield analysis was 4.5 m<sup>2</sup>. At each harvest two samples of about 300 g fresh material were taken for dry matter and for chemical analysis and stored in sealed plastic bags. All samples of aerial parts were stored at -24 °C until analysis.

#### Extraction of flavonol glycosides and phenolic acids

Fresh frozen aerial parts (approximately 2 g leaves and 1 g stalks) of stinging nettle were immediately homogenized in a centrifuge tube using an Ultra-Turrax (model T25; IKA Labortechnik, Staufen, Germany) for 1 min together with 20 mL aqueous 80 % MeOH followed by extraction for 2 h at room temperature. After the extraction the sample was filtered followed by filtration through a nylon 0.45 µm, Cameo 25P syringe filter and then analysed by RP-HPLC for flavonoids and phenolic acids. The efficiency and the reproducibility of the extraction procedure described above were determined by multiple extraction (3×20 mL 80 % MeOH) of fresh frozen leaves in quadruplicates which showed that extraction of 1×20 mL 80 % MeOH as described above ensured extraction of >98 % of the total flavonoids and phenolic acids and further the reproducibility of the extraction method (CV<5 %). For the determination of the efficiency of the extraction method the extract sample was centrifuged for 10 min using a Sorvall SA-600 head ( $G_{\max}$  20.845; Buch & Holm, Herlev, Denmark) between each extraction and the supernatant collected and analysed for flavonoids and phenolic acids. It should be noticed that it is very important that the fresh frozen leaves are extracted immediately in the organic solvent in order to avoid enzymatic degradation of flavonoids and phenolic acids in the plant material (data not shown).

#### Identification of flavonol glycosides and phenolic acids by LC-MS

Identification of flavonoids and phenolic acids in aqueous 80 % MeOH extracts of aerial parts of stinging nettle were performed on an Agilent HPLC-DAD-MS station (Agilent, Waldbronn, Germany) comprising of a series 1100 HPLC equipped with a diode array detector (DAD) and a LC/MSD SL detector fitted with an atmospheric pressure chemical ionization (APCI) source. Data acquisition and processing were performed using Agilent's ChemStation software. Separations were carried out on a Purospher® STAR reversed-phase (RP)-C18 end-capped column (5 µm particle size; 250×4.0 mm i.d., Merck,

Darmstadt, Germany) protected with a RP-18 guard cartridge (5 µm; 15×4 mm i.d., Merck, Darmstadt, Germany). The column temperature was maintained at 35 °C and the mobile phases consisted of solvent A (aqueous 1 % formic acid) and solvent B (100 % MeCN). Separations were performed by the following solvent gradient: 1 % B isocratic (2 min), 1 to 25 % B (28 min), 25 to 35 % B (10 min), 35 to 95 % B (5 min), 95 % B isocratic (5 min), 95 % B to 1 % B (1 min), 1 % B isocratic (9 min). All changes of solvent B were linear programmed. The flow rate was 0.4 mL min<sup>-1</sup> and the injection volume ranged from 1 to 10 µL.

UV-vis spectra of flavonoids and phenolic acids were recorded between 200 and 600 nm at a rate of 1.25 scans/sec and monitored at 280, 320, 360 and 500 nm. MS spectra were recorded consecutively in negative mode with the detector fitted with the APCI chamber. MS spectra were recorded in the range  $m/z$  50– $m/z$  1,000. The acquisition parameters were as follows: fragmentor 70 V; spray chamber parameters: nitrogen used as the drying gas at a flow of 7 l min<sup>-1</sup> and as nebulizing gas at temperature of 300 °C. A potential of 3 kV was used on the capillary and the corona current set to 15 µA. Flavonoids and phenolic acids were identified in *U. dioica* extracts based on comparison with authentic standards and/or tentatively identified based on their retention time and their LC-MS and UV-data (Table 1).

#### Quantification of flavonol glycosides and phenolic acids by RP-HPLC

Analytical RP-HPLC was performed on a Shimadzu HPLC LC-10 Series System equipped with a DAD detector (Hitachi L-7450). Flavonoids and phenolic acids were monitored at 280 and 360 nm and UV-spectra were recorded between 200–450 nm. Separations were performed on a LiChrospher® 100 RP-18 column (250×4.6 mm i.d., particle size 5.0 µm, Merck, Darmstadt, Germany) at 35 °C using the following solvent gradient: 0.5 % trifluoroacetic acid (TFA) in H<sub>2</sub>O–MeCN [0 min (90:10), 15 min (80:20), 25 min (70:30), 35 min (50:50), 45–60 min (10:90), 65–75 min (90:10)]. The flow rate was 1 ml min<sup>-1</sup> and the injection volume 20 µL. Acquisition off at 75 min. Flavonoids and phenolic acids were determined by external calibration curves of quercetin-3-*O*-rutinoside and 5-*O*-caffeoylquinic acid, respectively. Mean recovery rates (≈ accuracy) for 5-*O*-caffeoylquinic acid and quercetin-3-*O*-rutinoside, respectively, were >98 % with a relative standard deviation (RSD) of <5 %, and were determined by spiking a known amount of authentic standard of 5-*O*-caffeoylquinic acid and quercetin-3-*O*-rutinoside to *U. dioica* extract samples, respectively. The precision of the HPLC method was determined by four injections of a *U. dioica* extract sample in 1 day (intraday variation) and on four different days (interday variation). The overall intraday and interday variations were found to be less than 5 % for both flavonoids and phenolic acids.

#### Statistics

Analysis of variance were performed on each variable using the Statistical Analysis System (SAS Institute Inc., Cary, NC, USA). The variations (standard errors, SE), the significances of treatment effects (F-test) and regression

Table 1. Characteristic ions of phenolic acids and flavonoids in extracts of aerial parts of *Urtica dioica* as determined by LC-MS (APCI, Negative Mode, Capillary Voltage 70 V) and their UV-Spectra as determined by HPLC-DAD.

Peak <sup>a</sup>	R <sub>t</sub> (min) <sup>b</sup>	Compound	HPLC-DAD UV spectrum $\lambda_{\max}$ (nm)	LC-MS APCI $m/z$ (% base peak)
1	12.4	3- <i>O</i> -caffeoylquinic acid <sup>c</sup> (neochlorogenic acid)	298sh, 327	353 [M-H] <sup>-</sup> (100), 191 (1), 173 (2)
2	13.9	quercetin diglucoside	255, 264sh, 301sh, 350	625 [M-H] <sup>-</sup> (100), 463 (24), 301 (4)
3	14.6	5- <i>O</i> -caffeoylquinic acid <sup>c</sup> (chlorogenic acid)	300sh, 325	353 [M-H] <sup>-</sup> (100), 191 (15),
4	17.9	2- <i>O</i> -caffeoylmalic acid	302sh, 328	295 [M-H] <sup>-</sup> (100), 179 (8), 133 (16)
5	20.3	5- <i>O</i> -feruloylquinic acid	300sh, 324	367 [M-H] <sup>-</sup> (100), 191 (25)
6	21.8	unknown phenolic acid	300sh, 327	—
7	22.6	quercetin-3- <i>O</i> -rutinoside <sup>c</sup> (rutin)	256, 263sh, 298sh, 353	609[M-H] <sup>-</sup> (100), 463 (2), 301(2)
8	22.9	quercetin-3- <i>O</i> -glucoside <sup>c</sup> (isoquercitrin)	256, 263sh, 298sh, 353	463 [M-H] <sup>-</sup> (100), 301 (2)
9	24.4	kaempferol-3- <i>O</i> -rutinoside <sup>c</sup>	265, 299sh, 347	593 [M-H] <sup>-</sup> (100), 447 (1), 285(7)
10	25.3	isorhamnetin-3- <i>O</i> -rutinoside <sup>c</sup>	256, 268sh, 301sh, 352	623 [M-H] <sup>-</sup> (100), 315 (3)

<sup>a</sup>Peak numbers correspond to the peak numbers in Fig. 2. <sup>b</sup>R<sub>t</sub> = retention time on HPLC. <sup>c</sup>R<sub>t</sub>, UV and LC-MS data compared with an authentic standard.

coefficients were calculated and tested using the General Linear Models procedure and statistical differences were determined with the LSD test ( $P < 0.05$ ).

## Results and Discussion

### Flavonol glycosides and phenolic acids in stinging nettle

Phenolic constituents in extracts of aerial parts of *U. dioica* were monitored by RP-HPLC-DAD and LC-MS (APCI, negative mode). Typical HPLC chromatograms at 280 nm and 360 nm, respectively, of an aqueous MeOH extract of aerial parts of *U. dioica* are shown in Fig. 2. Based on the UV spectra the compounds could be grouped into (i) caffeic acid derivatives with an absorption band centred around 325 nm with a shoulder at around 299 nm and (ii) flavonol glycosides with the typical pattern of band I centred around 354 nm with a shoulder at around 299 nm and band II centred around 256 nm with a shoulder at around 264 nm (Table 1). The UV absorptions of compounds 1, 3–5 clearly indicated that these compounds were derivatives of caffeic acid, which was also confirmed by their LC-MS data. Compounds 1 and 3 had both a pseudomolecular ion [M-H]<sup>-</sup> at  $m/z$  353, compatible with caffeoylquinic acids and were identified as 3-*O*-caffeoylquinic acid and 5-*O*-caffeoylquinic acid, respectively, by comparison with authentic standards. These compounds have previously been reported as constituents in the aerial parts of *Urtica* species including *U. dioica* (BUDZIANOWSKI 1991; BUCAR et al. 2006). Compound 4 showed a pseudomolecular ion [M-H]<sup>-</sup> at  $m/z$  295 compatible with 2-*O*-caffeoylmalic acid, which was also confirmed by an ion at  $m/z$  179 corresponding to caffeic acid (FANG et al. 2002) and an ion at  $m/z$  133 corresponding to malic acid. 2-*O*-Caffeoylmalic acid has previously been identified as a major phenolic acid in the aerial parts of *U. dioica* (BUDZIANOWSKI 1991; BUCAR et al. 2006) in accordance with the results of the present study. Compound 5 gave a pseudomolecular ion [M-H]<sup>-</sup> at  $m/z$

367 and a major ion at  $m/z$  191 in accordance with this compound being 5-*O*-feruloylquinic acid which has also previously been identified in the aerial parts of *U. dioica* (BUDZIANOWSKI 1991).

Compounds 2, 7–10 showed typical UV spectra of flavonol glycosides, which were also confirmed by their LC-MS data. Compounds 2, 7 and 8 gave all an ion at  $m/z$  301 corresponding to the aglycone quercetin. Compounds 7 and 8 showed pseudomolecular ions [M-H]<sup>-</sup> at  $m/z$  463 and  $m/z$  609, respectively, and was identified as quercetin-3-*O*-glucoside and quercetin-3-*O*-rutinoside, respectively, by comparison with authentic standards. Compound 2 with a pseudomolecular ion [M-H]<sup>-</sup> at  $m/z$  625 showed also ions at  $m/z$  463 and 301 corresponding to the loss of a diosyl group or two glucose/galactose moieties. The relative high abundance of the ion at  $m/z$  463 indicated that it was not a dioside; hence compound 2 is a quercetin diglycoside (Table 1). Compounds 9 and 10 showed pseudomolecular ions [M-H]<sup>-</sup> at  $m/z$  593 and  $m/z$  623, respectively, and both the loss of 308 Da corresponding to the loss of a rutinoside moiety (Table 1). Consequently, compounds 9 and 10 were identified as kaempferol-3-*O*-rutinoside and isorhamnetin-3-*O*-rutinoside, respectively, and their identity was confirmed by comparison with authentic standards.

The flavonoids identified in the present investigation have all previously been found in the aerial parts of *U. dioica* (CHAURASIA and WICHTL 1987; BUDZIANOWSKI 1991; KAVTARADZE et al. 2001; AKBAY et al. 2003; BUCAR et al. 2006). However, the flavonoids isorhamnetin-3-*O*-glucoside and isorhamnetin-3-*O*-neohesperidoside previously reported to be present in the aerial parts of *U. dioica* (CHAURASIA and WICHTL 1987) were not detected in this investigation, which may be explained by genotypic differences.

### Effects of nitrogen fertilization and re-harvest on yield of aerial parts of stinging nettle

Higher nitrogen (N) application levels increased the yield of aerial parts substantially. The accumulated yield over

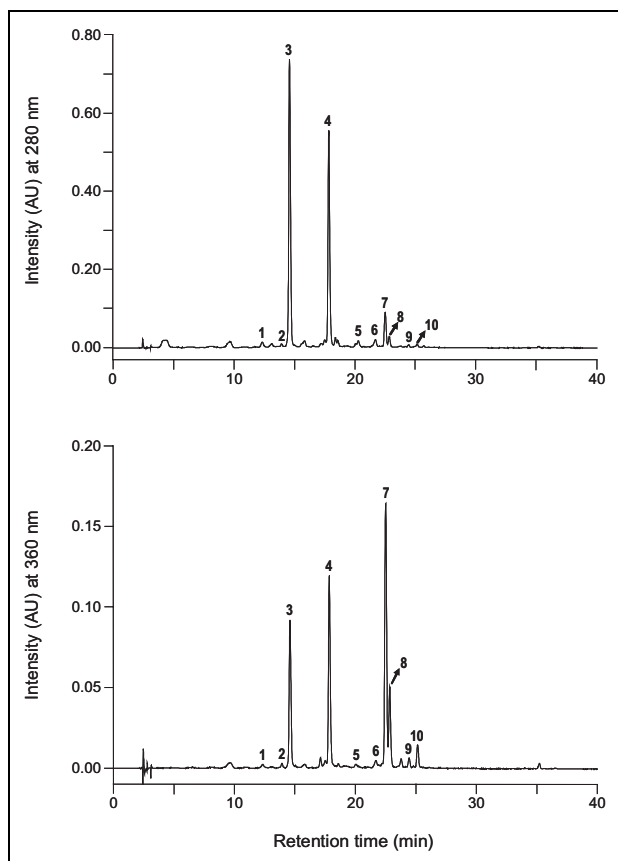


Fig. 2. Typical HPLC chromatograms of an aqueous 80% MeOH extract of aerial parts of stinging nettle (*Urtica dioica*) at 280 nm and 360 nm, respectively, showing the separation of flavonoids and phenolic acids in the extract. Compounds: 1 = 3-*O*-caffeoylquinic acid (neochlorogenic acid); 2 = quercetin diglucoside; 3 = 5-*O*-caffeoylquinic acid (chlorogenic acid); 4 = 2-*O*-caffeoylmalic acid; 5 = 5-*O*-feruloylquinic acid; 6 = unknown phenolic acid; 7 = quercetin-3-*O*-rutinoside (rutin); 8 = quercetin-3-*O*-glucoside (isoquercitrin); 9 = kaempferol-3-*O*-rutinoside; 10 = isorhamnetin-3-*O*-rutinoside (Fig. 1). The chromatographic conditions are described in Materials and Methods.

three years increased from 30 t ha<sup>-1</sup> at 0 kg N ha<sup>-1</sup> to 50.0, 68.4 and 87.6 t ha<sup>-1</sup> at 100, 200 and 400 kg N ha<sup>-1</sup>, respectively (Fig. 3). The exact harvest dates and yields in every single harvest are listed in Table 2. The 75 percent increase in accumulated yield over three years observed in our experiment going from 100 to 400 kg N ha<sup>-1</sup> is, however, considerably lower than the increase found in a similar study by WEISS (1993). WEISS (1993) found a yield increase of about 250 percent in going from 80 to 440 kg N ha<sup>-1</sup> also over a three year growing period and also as accumulated yield. The plants in the experiment by WEISS (1993) were established on similar soil type and with direct 'laying' of stolon cuttings in autumn and then four harvests of leaf and stalks per year. The reason for the difference in N-response is not clear but could be due to genotypic differences combined with a different plant density and/or climatic conditions. The potential yield of aerial parts seems to be far from reached in our experiment as

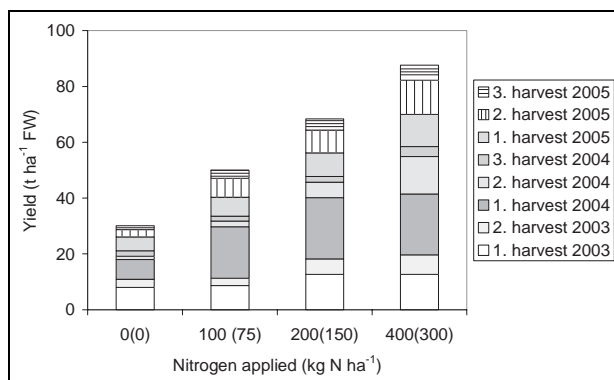


Fig. 3. The accumulated fresh weight yield of aerial parts (leaf and stalks) of stinging nettle (*Urtica dioica*) at four different nitrogen fertilizer applications. There were two or three harvests on the same plants per year in three years (2003–2005). Nitrogen applications in brackets are for 2003.

is the case in the study by WEISS (1993) where even the 440 kg N ha<sup>-1</sup> treatment did not show any 'tailing off' in the yield response curve. In our study the first harvest and second re-harvest (cut) in each year gave the highest yield (Fig. 3) whereas the third re-harvest may under our conditions not be profitable unless a very high N supply is given.

#### Effects of nitrogen fertilization and re-harvest on the content of flavonol glycosides and phenolic acids

Contrasting to yield of bio-mass higher N levels reduced the concentration of flavonol glycosides in the harvested plant material significantly, in almost all harvests (Table 2). If an average over six harvests in two years (2004 and 2005) is calculated there is a significant negative correlation ( $R^2 = 0.9973$ ,  $p = 0.014$ ) between N applied and concentration of total flavonol glycosides (Fig. 4). Results from year 2003 are omitted because of the difference in amounts of applied N and because this was the year of establishment with only two harvests. Year 2003 results show, however, also a significant lower concentration of flavonol glycosides in relation to higher N application (Table 2). The higher concentration of flavonol glycosides at low N levels may not be surprising because many of the flavonoids are biosynthesized as a reaction to stress conditions during growth (BONGUE-BARTELSMAN and PHILLIPS 1995; DIXON and PAIVA 1995; STEWART et al. 2001). A decline in content of flavonol glycosides with an increase in N supply (Fig. 4a) is in accordance with the results of similar studies performed on crops like apples (AWAD and DE JAGER 2002), strawberries (ANTTONEN et al. 2006) and tomatoes (STEWART et al. 2001). For example, ANTONEN et al. (2006) found that the lowest level of fertilization (not only N) in their experiment with strawberries increased the contents of flavonols in fruits by up to 57% compared to double the amount of fertilizer. STEWART et al. (2001) found that the 'flavonol' content was inversely related to nitrogen and phosphate nutrition of tomato, the effect of nitrogen being more pronounced than phosphate.

The effect of applied N on the concentration of phenolic acids did not show a similar clear correlation. In year

Table 2. Effects of nitrogen application and harvest date on fresh weight yield of aerial parts (leaf and stalk), dry matter content, total flavonoids and total phenolic acids as well as the content of major flavonoids (quercetin-*O*-3-rutinoside and quercetin-3-*O*-glucoside) and phenolic acids (5-*O*-caffeoylquinic acid and 2-*O*-caffeoylmalic acid) in stinging nettle (*Urtica dioica* L). The nettle crop was transplanted on May 23 2003 and harvested and re-harvested two or three times per year in the following three years. Data given as means (n=3 or 2) and values within the same column for each year and harvest date followed by different letters differ significantly at P<0.05.

Year	Harvest date	Nitrogen applied (kg N ha <sup>-1</sup> )	Yield (FW) (t ha <sup>-1</sup> )	Dry matter (DM) (%)	Total flavonoids	Quercetin-3- <i>O</i> -rutinoside	Quercetin-3- <i>O</i> -glucoside	Total phenolic acids	5- <i>O</i> -caffeoylquinic acid	2- <i>O</i> -caffeoylmalic acid
2003	5. July	0	8.0 a	19.1 a	4.8 a	3.2 a	1.4 a	20.7 a	4.1 a	14.9 a
		75	8.7 ab	19.0 a	3.7 ab	2.7 ab	0.9 a	11.5 b	3.1 a	7.1 b
		150	12.7 b	17.0 a	2.9 ab	2.1 ab	0.6 a	14.9 ab	3.5 a	10.4 ab
		300	12.7 b	17.4 a	2.4 b	1.7 b	0.6 a	12.5 b	2.5 a	9.1 b
	2. September	0	2.9 ab	26.6 a	10.0 a	7.4 a	2.3 a	13.4 b	5.7 b	6.0 a
		75	2.6 a	26.6 a	9.6 a	7.2 a	2.2 a	23.1 a	9.2 a	12.3 a
		150	5.6 ab	26.0 a	5.4 b	4.0 b	1.1 b	12.4 b	4.9 bc	6.4 b
		300	7.0 b	25.7 a	3.9 b	3.0 b	0.7 b	8.5 b	2.9 c	4.6 b
2004	26. May	0	7.1 a	21.7 a	13.5 a	8.3 a	4.8 a	38.7 a	14.6 a	21.4 a
		100	18.4 b	17.6 b	2.4 b	1.7 b	0.6 b	30.3 a	8.9 a	20.5 a
		200	21.9 c	17.2 b	3.9 b	2.2 b	1.3 b	30.8 a	10.5 a	19.1 a
		400	21.9 c	16.0 b	2.4 b	1.6 b	0.7 b	17.3 a	4.6 a	12.1 a
	16. July	0	1.2 a	24.3 a	12.5 ab	8.6 a	3.2 a	32.1 a	12.5 a	17.6 a
		100	2.1 a	24.3 a	14.5 a	9.8 a	4.3 a	35.2 a	16.3 a	16.8 ab
		200	5.6 b	22.3 a	8.4 b	5.2 b	2.8 ab	30.9 a	11.6 a	17.1 a
		400	13.3 c	19.3 b	2.7 c	1.9 c	0.6 b	20.5 b	5.7 b	14.0 b
	8. October	0	2.0 ab	25.9 a	7.7 a	5.9 a	1.5 a	31.8 a	14.6 a	16.7 a
		100	1.7 b	24.9 a	9.1 a	6.7 a	2.1 a	31.7 a	15.0 a	16.2 a
		200	2.0 ab	25.2 a	8.6 a	6.2 a	2.0 a	27.4 a	12.1 a	14.5 a
		400	3.6 c	25.7 a	8.4 a	6.1 a	1.9 a	29.0 a	11.7 a	16.6 a
2005	17. June	0	4.9 a	23.8 a	9.7 a	6.6 a	2.8 a	24.9 a	12.3 a	11.2 a
		100	6.8 ab	22.6 ab	10.8 a	7.1 a	3.5 a	28.8 a	13.6 a	13.7 a
		200	8.5 bc	21.8 bc	9.2 a	6.1 a	2.8 a	29.0 a	13.4 a	14.0 a
		400	11.6 c	20.8 c	7.5 a	4.8 a	2.5 a	26.8 a	11.5 a	13.9 a
	11. August	0	2.6 a	23.4 a	11.0 a	7.5 a	3.1 a	28.3 a	13.3 a	13.6 a
		100	6.8 ab	22.4 a	9.4 a	6.4 a	2.7 a	31.6 a	14.4 a	15.8 a
		200	8.2 bc	22.6 a	9.4 a	6.4 a	2.4 a	32.9 a	18.3 a	13.6 a
		400	12.2 c	21.2 b	4.7 a	3.2 a	1.2 a	22.4 a	10.3 a	11.2 a
	11. October	0	1.3 a	22.0 a	5.8 a	4.3 a	1.1 a	26.1 bc	8.5 ab	16.7 a
		100	2.9 ab	22.0 a	4.7 ab	3.6 ab	1.0 a	33.1 a	10.0 a	21.8 a
		200	4.0 bc	21.5 a	3.9 bc	2.8 bc	0.9 a	31.7 ab	9.2 ab	21.2 a
		400	5.4 c	22.6 a	2.8 c	2.1 c	0.6 a	24.7 c	7.4 b	16.4 a

2003 and 2004 there was a tendency to lower concentrations of phenolic acids with higher application of N, although only significant for the highest amount of N compared to the lowest (Table 2). However, in 2005 there was no significant effect of applied N on the content of phenolic acids in the harvested plant material. If again an average over six harvests in two years (2004 and 2005) is calculated there is a slightly negative correlation between concentration of phenolic acids and applied N, which, however, is not significant (Fig. 4b).

The first harvest time each year tended to be lower in concentration of flavonol glycosides but there was no significant difference in the concentration of phenolic acids over harvest times (Table 2). Looking at the single major flavonol glycosides as percentage of total flavonoids, the percentage of quercetin-3-*O*-rutinoside (rutin) was significantly increasing with later harvest time ( $p<0.001$ ) each year, in contrast to quercetin-3-*O*-glucoside (isouercitrin) whose relative concentration decreased with later harvest time (Fig. 5a). This result could indicate an

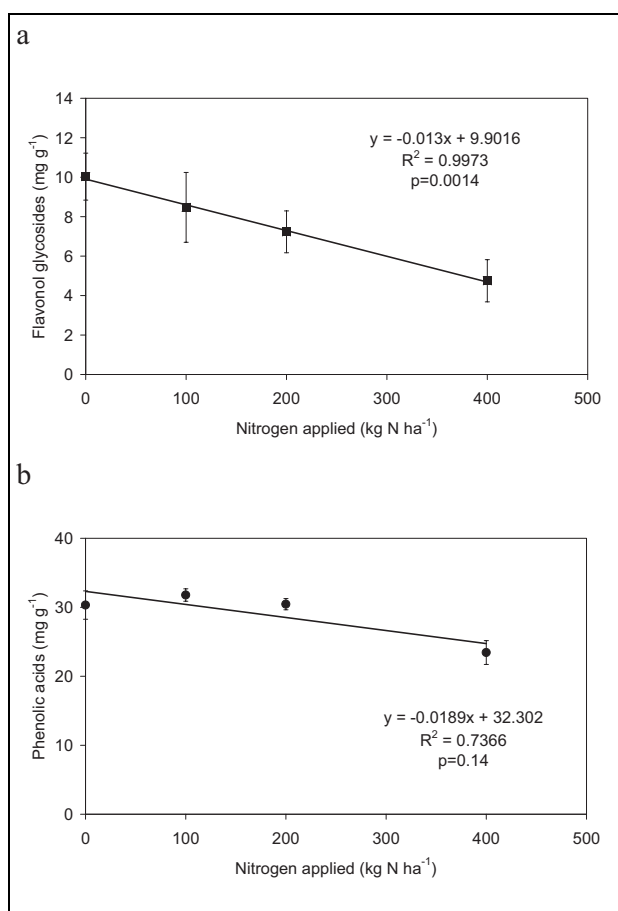


Fig. 4. Effects of nitrogen application on average concentration (mg g<sup>-1</sup> DM) of flavonol glucosides (a) and phenolic acids (b) in aerial parts of stinging nettle (*Urtica dioica*). Data are average of six harvests over two years (2004 and 2005). Bars are SE (n=6).

increasing anabolic activity towards the biosynthesis of quercetin-3-*O*-rutinoside from quercetin-3-*O*-glucoside with later harvest, although it cannot be excluded that the changes in the composition of the major flavonoids are due to a catabolic degradation of quercetin-3-*O*-glucoside and an increased anabolic activity towards the biosynthesis of quercetin-3-*O*-rutinoside. The percentage of 5-*O*-caffeoylquinic acid of the total phenolic acid concentration was a little higher in the second harvest each year ( $p=0.03$ ) and this was contrasted by a tendency to lower percentage of 2-*O*-caffeoylmalic acid in the second harvest ( $p=0.10$ ) (Fig. 5b). These results also suggest that the major phenolic acids undergo anabolic and catabolic changes during the growing season. For example, this could be a simple biosynthetic conversion of 2-*O*-caffeoylmalic acid into 5-*O*-caffeoylquinic acid by substituting malic acid with quinic acid.

The dry matter (DM) content of plant material (Table 2) is significantly negatively correlated to N application ( $R^2=0.9298$ ,  $p=0.036$ ) and decreases from an average of 23.5 % at 0 kg N ha<sup>-1</sup> to 21 % at 400 kg N ha<sup>-1</sup>. The theoretical yield of phenolics per area (calculated amounts of harvested phenolics in kg ha<sup>-1</sup>) were highest at an application rate of about 200 kg N ha<sup>-1</sup> for both flavonol glyco-

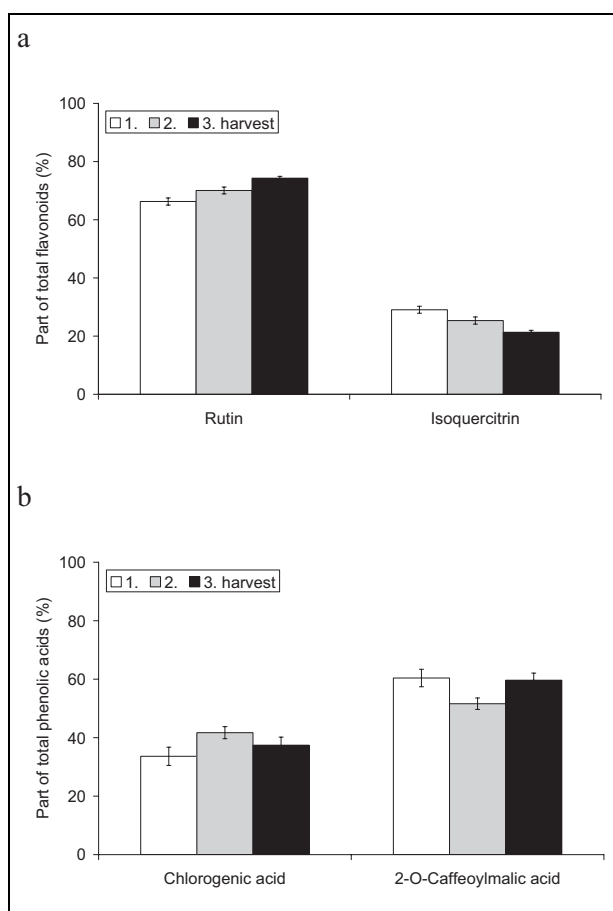


Fig. 5. Effects of harvest time (re-harvest on the same plants) on the composition of major flavonol glycosides (rutin and isoquercitrin) (a) and phenolic acids (chlorogenic acid and 2-*O*-caffeoylmalic acid) (b) in aerial parts of stinging nettle (*Urtica dioica*) as percent of total content of flavonoids and phenolic acids, respectively. Data are averages of four nitrogen applications over three years. Bars are SE (n=12 and n=8 in 3<sup>rd</sup> harvest).

sides and phenolic acids (Fig. 6) but the difference between N applications was not significant in either of them.

There was a high variation in the concentrations of phenolics over years, harvests and N applications (Table 2). In the study by WEISS (1993) the phenolics were not determined, but the concentrations found in the aerial parts of stinging nettle in the present investigation are comparable with the values found by SCHOMAKERS et al. (1995). They reported that the variation of quercetin-3-*O*-rutinoside in leaves of stinging nettle to be between 1 to 6 mg g<sup>-1</sup> DM and the variation of 2-*O*-caffeoylmalic acid to be between 0.3 to 16 mg g<sup>-1</sup> DM. The values in our experiment for quercetin-3-*O*-rutinoside varied from 1.5 to 9.8 mg g<sup>-1</sup> DM, and that of 2-*O*-caffeoylmalic acid between 4.6 to 21.8 mg g<sup>-1</sup> DM (Table 2) which is in a similar range as found by SCHOMAKERS et al. (1995). According to the EUROPEAN PHARMACOPOEIA (2007) the content of bioactive compounds in nettle leaf (*Urticae folium*) should be minimum 3 mg g<sup>-1</sup> DM expressed as the sum of 2-*O*-caffeoylmalic acid and chlorogenic acid, which is clearly below the amounts found in the present investigation (Table 2)



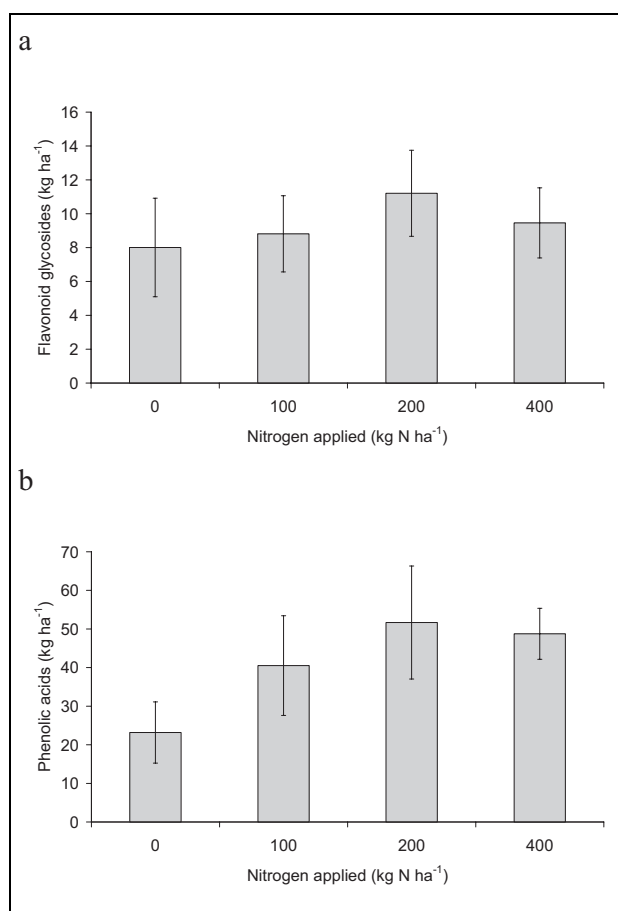


Fig. 6. Average potential yield per area of total flavonoids (a) and total phenolic acids (b) in aerial parts of stinging nettle (*Urtica dioica*) as an effect of applied nitrogen. Data are averages of six harvests over two years (2004 and 2005). Bars are SE (n=6).

The results of the present study may be used to optimise the yield and quality of a stinging nettle product and to lower the cost of cultivation and processing. However, the results of the present investigation clearly shows that cultivation of *U. dioica* herba for a high yield of bioactive compounds is a compromise between a high yield of plant material and the content of flavonol glycosides and phenolic acids in the harvested product.

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