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Content and Composition of Volatile Terpenes, Flavonoids and Phenolic Acids in Greek Oregano (*Origanum vulgare* L. ssp. *hirtum*) at different Development Stages during Cultivation in Cool Temperate Climate

K. Grevsen[1)], X.C. Fretté[2)] and L.P. Christensen[2)]
([1)]Department of Horticulture, Faculty of Agricultural Sciences, University of Aarhus, Aarslev, Denmark, [2)]Institute of Chemical Engineering, Biotechnology and Environmental Technology, Faculty of Engineering, University of Southern Denmark, Odense, Denmark)

Summary

Plants of *Origanum vulgare* ssp. *hirtum* (Greek oregano) were cultivated in two years in a cool temperate climate and the aerial parts were harvested at up to five different development stages during the growing season (July to late August) in order to investigate how the content of volatile terpenes and polyphenols depends on the development stage of the plants. Volatile terpenes were identified and quantified in dichloromethane extracts by GC-MS and GC-FID, respectively. Polyphenols were quantified in aqueous methanol extracts by RP-HPLC-PDA and identified by LC-PDA-MS and NMR spectroscopy. Significant changes in the concentrations and composition of major volatile terpenes (carvacrol, γ-terpinene, *p*-cymene, myrcene, α-pinene, β-caryophyllene, thymol) were observed at the different development stages. The content of volatile terpenes varied from 3.7–4.9 % (year 1) and 2.6–4.6 % (year 2). The potential yields of total volatile terpenes varied from 74–165 kg ha^{-1} (year 1) and 31–79 kg ha^{-1} (year 2) being highest near full flowering stage (50–60 % open flowers). Major polyphenols were eriodictyol 6,8-di-*C*-glucoside, apigenin 6,8-di-*C*-glucoside, luteolin 7-*O*-glucuronide, *epi*-lithospermic acid B, lithospermic acid B and rosmarinic acid. The content of flavonoids varied from 4.8–7.7 mg g^{-1} dry matter (DM) (year 1) and 8.9–12.4 mg g^{-1} DM (year 2), and phenolic acids from 1.4–10.1 mg g^{-1} DM (year 1) and 23.4–51.5 mg g^{-1} DM (year 2). The highest content of flavonoids in aerial parts was obtained near full flowering stage and for phenolic acids earlier at around 10–20 % open flowers. The conclusion of this study is that also in a cool temperate climate the development stage has a significant impact on the content and composition of both volatile terpenes and polyphenols in *O. vulgare* ssp. *hirtum*, and that an optimal harvest time of aerial parts of this plant depends on the secondary metabolites of interest.

Key words. Greek oregano – phenological development – terpenes – carvacrol – thymol – flavonoid glycosides – rosmarinic acid

Introduction

Origanum vulgare L. ssp. *hirtum* (Greek oregano, *Lamiaceae*) is a perennial herb widely distributed in Eurasia and North Africa. It is commonly used throughout the Mediterranean regions as a spice under the name "oregano" and from a morphological point of view it can be distinguished by its small green bracts and white flowers. Oregano is also commonly used as a medicinal plant with powerful antimicrobial activity (Adam et al. 1998; Daferera et al. 2000; Aligiannis et al. 2001; Elgayyar et al. 2001; Chorianopoulos et al. 2004) and antioxidant activity (Kikuzaki and Nakatani 1989; Chun et al. 2005; Cavero et al. 2006). The antimicrobial activity of *O. vulgare* ssp. *hirtum* is mainly due to its high concentration of essential oils (about 5 % in DM), which normally contains the monoterpenes carvacrol and thymol, accompanied by their biosynthetic precursors *p*-cymene and γ-terpinene as the primary components (Fig. 1) (Kokkini et al. 1997; Russo et al. 1998; Skoula et al. 1999; Chorianopoulos et al. 2004). Most of the cultivated *O. vulgare* ssp. *hirtum* types seem to be of the carvacrol chemotype with up to 80 % carvacrol in the essential oil. The closely related *O. vulgare* ssp. *vulgare* (wild marjoram) on the other hand is lower in its concentration of volatile terpenes (up to 1 % in DM) and with a different volatile terpene profile consisting of medium to high thymol/carvacrol content as well as relative high amounts of sabinene and γ-terpinene and the sesquiterpenes cubebene, germacrene, and β-caryophyllene (Skoula et al. 1999; Röhricht and Mänicke 2004).

The antioxidant activity of oregano is mainly due to flavonoids and phenolic acids (Kikuzaki and Nakatani

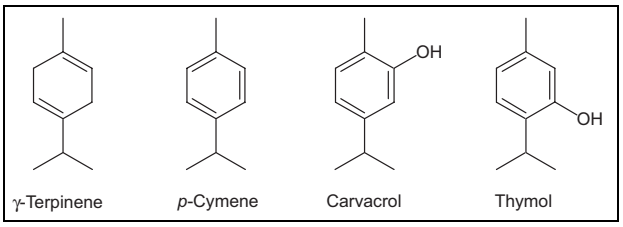


Fig. 1. Typical main volatile terpenes present in aerial parts of *Origanum vulgare* ssp. *hirtum*.

1989; CHUN et al. 2005; CAVERO et al. 2006). Rosmarinic acid and derivatives of lithospermic acid and the flavones apigenin and luteolin appear to constitute the main groups of polyphenols in oregano (Fig. 2) (LIN et al. 2003; CAVERO et al. 2006; KOUKOULITSA et al. 2006a, b). Dietary phenolic antioxidants play important roles in delaying the development of chronic diseases such as cardiovascular diseases, inflammatory bowel syndrome and Alzheimer's diseases and hence polyphenols are important for the health effects of oregano (SHETTY 1997; RÖHRICHT and MÄNICKE 2004). The aim of the present study was to investigate how the content of volatile terpenes, flavonoids and phenolic acids in *O. vulgare* ssp. *hirtum*, depend on the development stage during the growing season in a cool temperate climate zone.

We reasoned that the development stage of *O. vulgare* ssp. *hirtum* at harvest may influence the concentration and composition of volatile terpenes, flavonoids and phenolic acids in aerial parts, and hence the quality of the harvested plant material. To test this hypothesis, we harvested aerial parts of *O. vulgare* ssp. *hirtum* up to five succeeding times in two years and sampled the material for analyses of yield, dry matter and concentration and composition of volatile terpenes, flavonoids and phenolic acids.

Materials and Methods

Plant material

The plant material of *O. vulgare* ssp. *hirtum* used in the present investigation was originally obtained from the Uni-

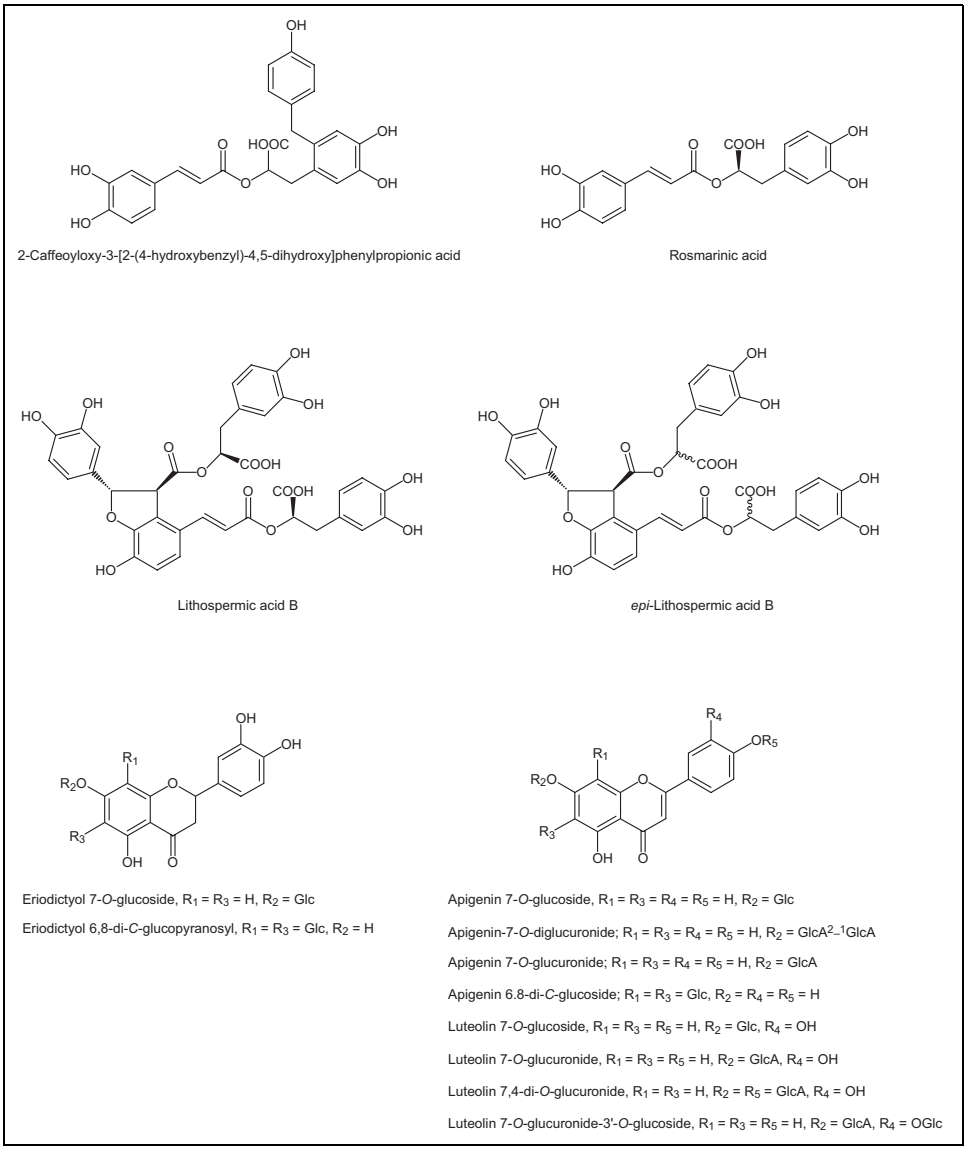


Fig. 2. Flavonoids and phenolic acids identified in aerial parts of *Origanum vulgare* ssp. *hirtum* in the present investigation. Glc = glucose; GlcA = glucuronic acid.

versity of Massachusetts, Department of Food Science, U.S.A. by a Danish herb grower (Martin Jørgensen, Biosynergy A/S). The transplants for the crop were propagated by tissue culture from a clonal line of oregano (clonal line O-1) that was previously generated from a single seedling (Shetty et al. 1995). The selected plant was propagated *in vitro* first by auxiliary bud proliferation and then later by simple division. The plants in the field used for the experiments were therefore genetically identical.

Field experiments

The *O. vulgare* ssp. *hirtum* crop used for harvest in the 2003 experiment was planted in 1999 by means of transplants in large speedling pots on a sandy loam soil at location Guldbjerg (10° 10' E, 55° 33' N) and the crop for harvest in 2004 was grown on a clay loam soil at location Barritsskov (9° 50' E; 55° 43' N). The plants were organic grown and received about 75 kg N ha^{-1} in the form of chicken manure (Binadan A/S, Nørre Snede, Denmark) every second year. In the year of sampling the plants did not receive manure. The plants were established in a bed system using black woven plastic (MyPex) ground cover to control weeds. The individual beds were 1.5 m wide. The woven plastic cover had pre-punched holes to allow mechanical planting. The plant density in beds was 16 plants m^{-2} in four rows and with a row distance of 0.3 m and an in-row spacing of 0.2–0.25 m. The plastic cover was 1.5 m in width and secured with soil along the edges. The spacing between individual beds was 1.0 m wide. In the space between plots, a grass mixture was sown and the grass in these passageways was kept short by frequent moving.

The individual plot size for the different harvest times in 2003 and 2004 was 2 m and 1.5 m long beds, respectively. The whole bed width was harvested and the area for yield calculation was 3 m^2 in 2003 and 2.3 m^2 in 2004. The statistical design of the field experiment was in randomized blocks with three replicates of the harvest time treatment in both 2003 and 2004.

Harvest time and re-harvest

The aerial shoots of the plots were harvested at five different development stages in 2003 and at four stages in 2004 (July to August). In 2003, the plots for the second harvest were re-harvested in October to investigate the possibility for two harvests per season and how this affects the content of bioactive compounds. The harvests were timed to be before and after full flowering, and full flowering was near the third harvest in each year. The shoots (stems, leaves and flowers) were cut off about 5 cm over ground with a modified hedge cutter and the yield of fresh material was recorded by weighing just after cutting. At each harvest time, two samples of about 300 g were collected, weighed, and packed in sealed airtight plastic bags for dry matter analysis and for analysis of polyphenols. A smaller sample of about 30 g was collected and placed in an airtight glass bottle (Blue cap, 100 ml) for analysis of volatile terpenes. All samples were kept deep frozen at −24 °C until analyses.

Chemicals

Methanol (MeOH), dichloromethane (CH_2Cl_2), acetonitrile (MeCN), acetic acid ($MeCO_2H$) 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, eriodictyol, eriodictyol 7-*O*-glucoside, apigenin 7-*O*-glucoside, and luteolin 7-*O*-glucoside were purchased from Extrasynthese (Genay, France). Rosmarinic acid, lithospermic acid B, apigenin 6,8-di-*C*-glucoside (vicenin-2) and apigenin 7-*O*-diglucuronide were isolated from a MeOH extract of *O. vulgare* ssp. *hirtum* by column chromatography and preparative HPLC and identified by nuclear magnetic resonance (NMR) spectroscopy and LC-PDA-MS and used as authentic standards (Table 1). NMR spectra were recorded at 500 or 300 MHz in d_4-CD_3OD with trimethylsilane (TMS) as internal standard. Volatile terpenes (Fig. 3) were purchased from Aldrich-Chemie or TCI Tokyo Organic Chemicals (Tokyo, Japan).

Extraction of volatile terpenes

Volatile terpenes were extracted from fresh frozen aerial parts of *O. vulgare* ssp. *hirtum* (0.5 g) with CH_2Cl_2 (5 mL) for 20 h at 5 °C. After filtration 10 μL 4-methyl-1-pentanol (8140 ng μL^{-1}) was added as internal standard. Each sample of 0.5 g was taken from a representative sample of 30 g aerial parts that was homogenized in liquid nitrogen. The extraction of volatile terpenes was performed in triplicates and the extraction procedure resulted in almost complete extraction (> 95 %) of volatile terpenes as shown by sequential extraction experiments on the same plant material (data not shown).

Identification of volatile terpenes by GC-MS

A Varian Saturn 2000 ion trap mass spectrometer operated at an ionization potential of 70 eV and directly coupled to a Varian Star 3400 CX gas chromatograph (Varian Palo Alto, CA) equipped with a split/splitless injector (split ratio 1:50) was used. Volatile terpenes were separated on a Chrompack (Middleburg, The Netherlands) WCOT fused silica capillary column (50 m×0.25 mm i.d., DF = 0.2 μm liquid phase: CP-Wax 52CB) using the following temperature program: 33 °C for 1 min, followed by 2 °C min^{-1} to 130 °C, 3.5 °C min^{-1} to 160 °C, 8 °C min^{-1} to 220 °C followed by constant temperature for 10 min. Helium was applied as a carrier gas at a flow rate of 1.4 mL min^{-1} and 20 psi column head pressure. Injection volume: 1 μl. The temperature of the injector and the transfer line was 200 °C. The mass spectrometer was operated in scan mode over a mass range from 39 to 350 amu (1 scan s^{-1}). Compounds suggested by the NIST MS database (Nist 1998) were verified by comparison of GC retention indices (RI) (Van Den Dool and Kratz 1963) and mass spectra of authentic reference compounds (Fig. 3).

Quantification of volatile terpenes by GC-FID

A Hewlett-Packard 5890 Series II Plus gas chromatograph (Hewlett-Packard, Avondale, PA) equipped with a split/splitless injector (200 °C) and a flame ionization de-

Table 1. Characteristic ions of flavonoids and phenolic acids in extracts of aerial parts of *Origanum vulgare* ssp. *hirtum* as determined by LC-MS (APCI, Negative Mode, Capillary Voltage 200 V) and their UV Spectra as determined by HPLC-PDA.

Compound number[a]	R_t (min)[b]	Compound	HPLC-PDA UV spectrum λ_{max} (nm)	LC-MS APCI *m/z* (% base peak)
1	11.7	Unknown flavanone	284, 324sh[c]	—[d]
2	13.7	2-Caffeoyloxy-3-[2-(4-hydroxybenzyl)-4,5-dihydroxy]phenylpropionic acid[e]	298sh, 323	465 $[M-H]^-$ (56), 303 (14), 277 (12), 179 (100)
3	14.5	Eriodictyol 6,8-di-*C*-glucoside[e]	291, 324sh	611 $[M-H]^-$ (55), 593(100), 575(59), 557(28), 503(48), 491(88), 485(21), 473(57), 455(8), 401(13), 371(5), 287(1)
4	20.8	Eriodictyol 7-*O*-glucoside[f]	284, 325sh	449 $[M-H]^-$ (100), 287 (5)
5	22.6	Apigenin 6,8-di-*C*-glucoside (vicenin-2)[g]	271, 335	593 $[M-H]^-$ (100), 575(27), 557(12), 503(52), 485(14), 473(70), 467(3), 455(7), 437(2), 413(6), 383(8), 353(4), 269(1)
6	24.1	Unknown flavanone	284, 324sh	—
7	27.1	Luteolin 7,4'-di-*O*-glucuronide[e]	257, 264sh, 346	637 $[M-H]^-$ (30), 461(100), 351(47), 285(82)
8	29.3	Unknown phenolic acid	300sh, 312	—
9	30.5	Luteolin 7-*O*-glucuronide-3'-*O*-glucoside[e]	258, 265sh, 287sh, 347	623 $[M-H]^-$ (100), 461(23), 447(3), 337(23), 285(73)
10	31.5	Apigenin 7-*O*-diglucuronide[g]	267, 336	621 $[M-H]^-$ (72), 449(43), 445(76), 431(87), 351(61), 269(100)
11	33.7	Luteolin 7-*O*-glucuronide[e]	255, 265sh, 347	461 $[M-H]^-$ (100), 285 (68)
12	33.9	Luteolin 7-*O*-glucoside[f]	254, 265sh, 347	447 $[M-H]^-$ (100), 285 (2)
13	34.7	Apigenin 7-*O*-glucoside[f]	266, 337	431 $[M-H]^-$ (100), 269(6)
14	35.9	Apigenin 7-*O*-glucuronide[e]	266, 336	445 $[M-H]^-$ (100), 269(54)
15	37.4	Unknown flavanone	284, 325sh	—
16	38.2	Rosmarinic acid[g]	294sh, 329	359 $[M-H]^-$ (100), 197(28), 179(8), 161(46)
17	40.6	Unknown flavone	266, 336	—
18	41.6	*epi*-Lithospermic acid B[e]	254, 286, 308sh, 330sh	717 $[M-H]^-$ (100), 537(12), 519(8), 465(6), 339(15), 243(8)
19	45.3	Lithospermic acid B[g]	254, 286, 308sh, 330sh	717 $[M-H]^-$ (100), 537(15), 519(13), 493(6), 465(6), 339(15), 295(3), 267(7), 243(7)

[a]Compound numbers also correspond to the peak numbers in Fig. 4. [b]R_t = retention time on HPLC. [c]sh = shoulder. [d]No characteristic or conclusive LC-MS data obtained. [e]Identification based R_t, UV and LC-MS data compared with data from the literature (Parejo et al. 2004; Koukoulista et al. 2006a, b). [f]Identified by comparison with an authentic standard. [g]Compound isolated and identified by UV, LC-MS and NMR.

tector (FID) operating at 230 °C was used for quantification of volatile terpenes. Volatile terpenes were analysed under the same conditions (column, carrier gas flow, temperature program, injection volume) as described above for the GC-MS analysis. The individual volatiles were quantified from the FID peak areas relative to that of the internal standard (4-methyl-1-pentanol). The response factor was set to 1 for all compounds. The precision of the GC-FID method used for quantification of volatile terpenes was determined by four injections of an *O. vulgare* ssp. *hirtum* extract sample within one day (intraday variation) and on four different days (interday variation). The overall intraday and interday variations were less than 5 % for all major volatile terpenes. Mean recovery rates for major volatile terpenes such as carvacrol, *p*-cymene, and γ-terpinene were > 92 % with a relative standard deviation (RSD) of < 5 %, and were determined by spiking a known amount of authentic standard of carvacrol, *p*-cymene, and γ-terpinene, respectively, to *O. vulgare* ssp. *hirtum* extract samples.

Extraction of polyphenols

Three g fresh frozen aerial parts of *O. vulgare* ssp. *hirtum* were immediately homogenized in a centrifuge tube (40 mL) using an Ultra Turrax blender (model T25; IKA Labortechnik, Staufen, Germany) for 1 min together with 20 mL 80 % aqueous MeOH followed by extraction for 2 h at room temperature. After extraction, the sample extract was centrifuged for 10 min using a Sorvall SA-600 head (G_{max} 20.845; Buch & Holm, Herlev, Denmark) and the supernatant collected followed by filtration through a nylon 0.45 µm, Cameo 25P syringe filter into a HPLC vial and then analysed by RP-HPLC for flavonoids and phe-

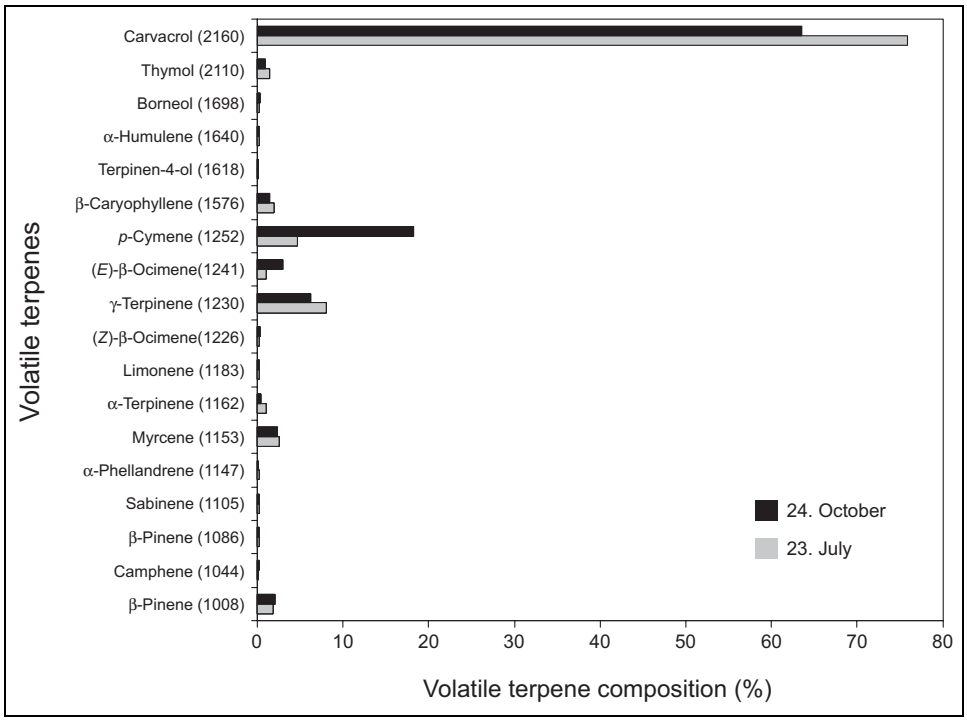


Fig. 3. Effects of development stage at harvest on composition (%) of volatile terpenes in aerial parts of *Origanum vulgare* ssp. *hirtum*. The columns show 2nd harvest time 23 July and re-harvest 24 October in 2003 (see Table 2 for details). Numbers in parentheses are the retention indices for each compound as determined by GC-FID (see Materials and Methods).

nolic acids. This extraction method ensured almost complete extraction (> 95 %) of flavonoids and phenolic acids as shown by sequential extraction experiments on the same plant material (data not shown). All extractions were made in duplicates.

Identification of polyphenols by LC-PDA-MS

Identification of flavonoids and phenolic acids in extracts of aerial parts of *O. vulgare* ssp. *hirtum* were performed on an Agilent HPLC-PDA-MS station (Agilent, Waldbronn, Germany) comprising of an Agilent 1100 Serries LC/MSD system equipped with a photodiode array (PDA) detector and a LC/MSD SL quadrupole mass spectometer 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 Luna reverse phase (RP)-C18 column (3 μm particle size; 150×2.0 mm i.d., Phenomenex, Torrance, CA, USA). 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: 0–30 min 1 % B, 70 min 25 % B, 80 min 35 % B, 85 min 95 % B, 90 min 95 % B, 95–100 min 1 % B. The flow rate was 0.2 mL min^{-1} and the injection volume was 2 μL.

UV-vis spectra of flavonoids and phenolic acids were recorded between 200 and 600 nm and monitored at 280, 320, 360 and 500 nm. MS spectra were recorded in negative mode with the detector fitted to the APCI chamber. The acquisition parameters were as follows: fragmentor 70 or 200 V; spray chamber parameters: N_2 used as the drying gas at a flow of 12 L min^{-1} and as nebulizing gas at a pressure of 45 psi and temperature of 300 °C. A potential of 3 kV was used on the capillary and the corona current set to 15 μA. Identification of flavonoids and phenolic acids in *O. vulgare* ssp. *hirtum* extracts were based on LC-MS, NMR and UV data as described in Table 1.

Quantification of polyphenols by RP-HPLC-PDA

Analytical RP-HPLC was performed on a Shimadzu HPLC LC-10 Series System equipped with a PDA detector (Hitachi L-7450). Flavonoids and phenolic acids were monitored at 280 and 360 nm and UV spectra were recorded between 200 and 400 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: Solvent A (aqueous 5 % formic acid) and Solvent B (50 % MeOH–15 % $MeCO_2H$–35 % H_2O). Gradient: 0 min 10 % B, 10 min 23 % B, 25 min 35 % B, 35 min 40 % B, 65 min 100 % B, 67 min 100 % B, 70–80 min 10 % B. The flow rate was 1 mL min^{-1} and the injection volume 20 μL. Polyphenols were determined by external calibration curves of 5-*O*-caffeoylquinic acid (phenolic acids), eriodictyol (flavanones) and luteolin (flavones), respectively. Further, the molecular weight of the identified compounds was included in the calculations of their concentration. The concentration of non-identified phenolic acids, flavanones and flavones were determined directly from the external calibration curves. Mean recovery rates for the external standards were > 98 % with a RSD of < 5 %, and were determined by spiking a known amount of authentic standard of 5-*O*-caffeoylquinic acid, eriodictyol and luteolin to *O. vulgare* ssp. *hirtum* extract samples, respectively. The precision of the HPLC method was determined by four injections of an *O. vulgare* ssp. *hirtum* extract sample within one day (intraday variation) and on four different days (interday variation). The overall intraday and interday variations were < 5 % for both flavonoids and phenolic acids.

Statistics

Analysis of variance was performed on each variable using the Statistical Analysis System (SAS Institute Inc., Cary, NC, USA). The variations (standard error of means, SE), the significances of treatment effects (F-test) and regression coefficients were calculated and tested using the General Linear Models procedure of SAS. Mean values of treatments in tables were separated by Duncan's multiple range test ($P < 0.05$).

Results and Discussion

Volatile terpenes

Eighteen volatile terpenes were detected repeatedly and quantified in CH_2Cl_2 extracts of aerial parts of *O. vulgare* ssp. *hirtum* (Fig. 3). The terpenes were identified by comparison of their mass spectral data with those of authentic reference compounds (see Materials and Methods) and accounted for > 98 % of the total volatile terpene mass in the extracts. Monoterpenes were the predominant class of volatile terpenes in terms of number and amounts with carvacrol and its biosynthetic precursor's γ-terpinene and *p*-cymene as the most abundant terpenes accounting for approximately 90 % of the identified terpenes (Fig. 3). The thymol content was low (0.4–1.5 %) indicating that the *O. vulgare* ssp. *hirtum* clone used here was of the carvacrol chemotype (Russo et al. 1998). Sesquiterpenes such as α-humulene and β-caryophyllene accounted for less than 3 % of the identified terpenes. The identified volatile terpenes have previously been isolated from the essential oils of *O. vulgare* ssp. *hirtum* (Kokkini 1997; Russo et al. 1998; Chorianopoulos et al. 2004; Wolff et al. 2007).

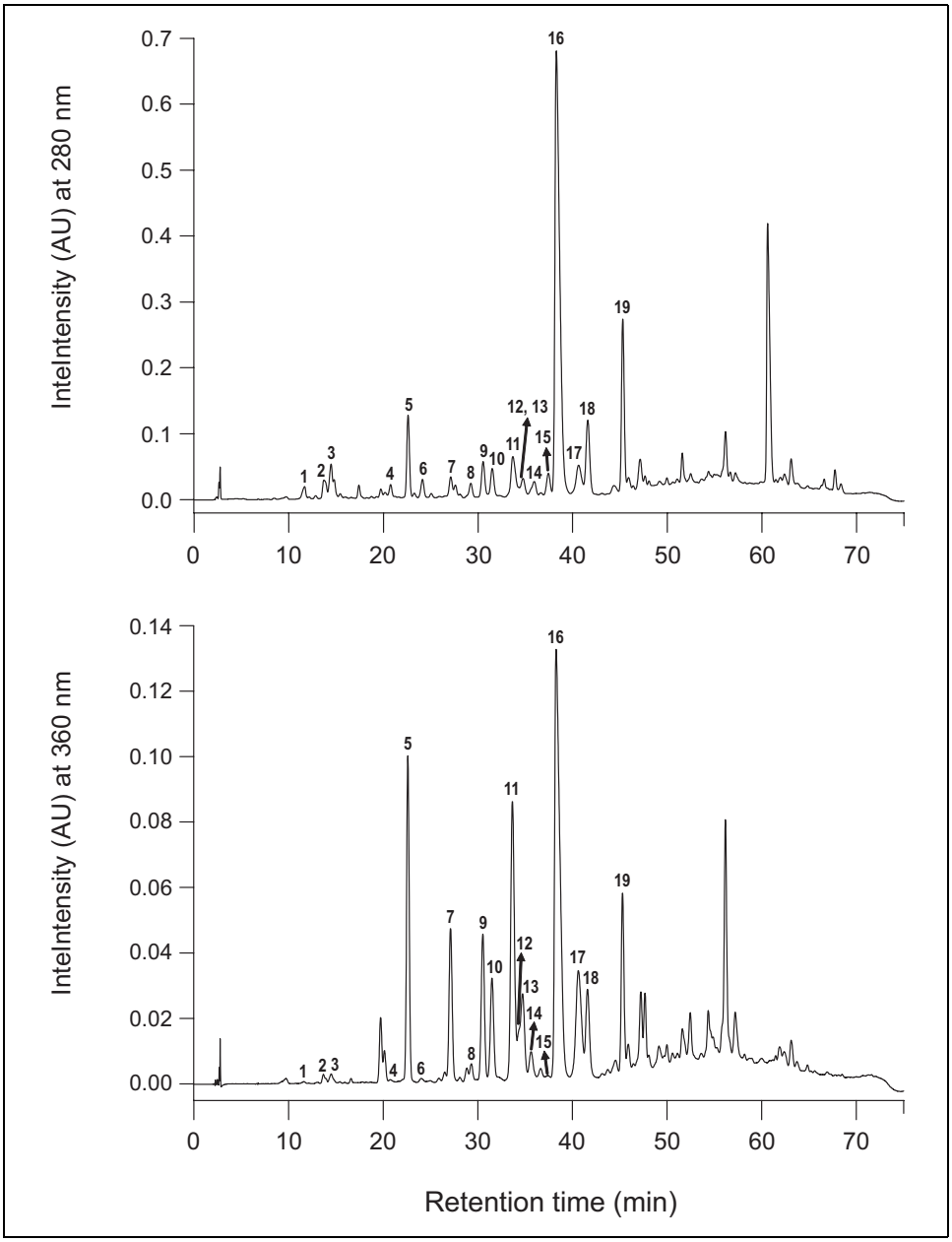


Fig. 4. Typical HPLC chromatograms of an aqueous 80 % MeOH extract of aerial parts of *Origanum vulgare* ssp. *hirtum* at 280 nm and 360 nm, respectively, showing the separation of flavonoids and phenolic acids in the extract. Identity of peak numbers (compound numbers) 1–19 are given in Table 1. The chromatographic conditions are described in Materials and Methods.

Flavonoids and phenolic acids

Polyphenols in extracts of aerial parts of *O. vulgare* ssp. *hirtum* were monitored by RP-HPLC-PDA and LC-MS (AP-CI, negative mode). Typical HPLC chromatograms at 280 nm and 360 nm, respectively, of an 80 % aqueous MeOH extract of aerial parts of *O. vulgare* ssp. *hirtum* are shown in Fig. 4. The identified and quantified polyphenols were caffeic acid derivatives, flavones, flavanones and lithospermic acid derivatives (Fig. 2 and Table 1).

The UV spectra of compounds **2** and **16** with an absorption band centred around 325 nm and with a shoulder at around 298 nm clearly indicated that these compounds were caffeic acid derivatives (Table 1). This was confirmed by their LC-MS data as both compounds showed an ion at *m/z* 179 corresponding to caffeic acid (Fang et al. 2002). Compound **16** had a pseudomolecular ion $[M-H]^-$ at *m/z* 359 indicating that this compound is rosmarinic acid, previously isolated from oregano (Koukoulista et al. 2006a, b). The identity of rosmarinic acid was confirmed by NMR analysis. Compound **2** showed a $[M-H]^-$ at *m/z* 465 being 106 Da higher than that of rosmarinic acid, corresponding to a hydroxybenzyl moiety. Therefore, **2** was tentatively identified as 2-caffeoyloxy-3-[2-(4-hydroxybenzyl)-4,5-dihydroxy]phenylpropionic acid, a rosmarinic acid derivative also previously isolated from oregano (Kikuzaki and Nakatani 1989).

Compounds **5**, **10**, **13** and **14** showed typical UV spectra for apigenin derivatives with band I centred around 336 nm and band II centred around 266 nm, which was also confirmed by their LC-MS data showing an ion at *m/z* 269 corresponding to the aglycone apigenin (Table 1). Compound **5** showed a complex fragmentation pattern with loss of water $[M-H-18]^-$ and E_1^- ($[M-H-36]^-$), E_2^- ($[M-H-120]^-$), $^{0,2}X^-$ ($[M-H-120]^-$) and $^{0,3}X^-$ ($[M-H-90]^-$) fragments (Cuyckens et al. 2000; Waridel et al. 2001). Fragmentation patterns with relatively abundant X ions in the negative ion mode, as observed in the LC-MS spectrum of **5** (Table 1), has previously been described as characteristic of *C*-glucoside flavonoids (Cuyckens et al. 2000; Waridel et al. 2001). Furthermore, the fragments $[M-H-240]^-$ and $[M-H-180]^-$ suggest a di-*C*-glucosyl substitution. Because common known glycosylation positions for flavones are on carbons 6 and/or 8 (Cuyckens et al. 2000; Waridel et al. 2001), **5** was identified as apigenin 6,8-di-*C*-glucoside (vicenin-2), which has recently been isolated from *O. vulgare* ssp. *hirtum* (Koukoulista et al. 2006a, b). The identity of **5** being apigenin 6,8-di-*C*-glucoside was confirmed by NMR analysis. The apigenin derivatives **13** and **14** showed a $[M-H]^-$ at *m/z* 431 and 445, respectively, and fragment ions corresponding to the loss of a glucose (($[M-H-162]^-$) or a glucuronic acid (($[M-H-176]^-$) moiety. The substitution of a glucose or glucuronic acid typically occurs at C7-OH in flavonoids (Cuyckens et al. 2000). Consequently, **13** and **14** were identified as apigenin 7-*O*-glucoside and apigenin 7-*O*-glucuronide, respectively, by comparison with an authentic standard and/or literature data (Table 1). The apigenin derivative **10** with an $[M-H]^-$ at *m/z* 637 gave fragment ions at *m/z* 445 and 269 corresponding to the loss of two glucuronic acid moieties. Hence, **10** was identified as apigenin-7-*O*-diglucuronide, which was also confirmed by NMR analysis. Compounds **5** and **14** have previously been isolated from *O. vulgare* ssp. *hirtum* (Lin et al. 2003; Koukoulista et al. 2006a, b) whereas compounds **10** and **13** are reported here for the first time from *O. vulgare* subspecies.

Compounds **7**, **9**, **11** and **12** showed typical UV spectra for luteolin derivatives with band I centred around 347 nm and band II centred around 256 nm with a shoulder at around 265 nm. This was confirmed by their LC-MS data showing an ion at *m/z* 285 corresponding to the aglycone luteolin. Compounds **11** and **12** gave a $[M-H]^-$ at *m/z* 461 and 447, respectively, and fragments corresponding to the loss of a glucose or a glucuronic acid moiety. Consequently, **11** and **12** were identified as luteolin 7-*O*-glucuronide and luteolin 7-*O*-glucoside, respectively, which was also confirmed by comparison with an authentic standard and/or data from the literature (Table 1). Luteolin 7-*O*-glucuronide and luteolin 7-*O*-glucoside have previously been isolated from *O. vulgare* (Lin et al. 2003). Compound **7** with $[M-H]^-$ at *m/z* 637 showed fragment ions at *m/z* 461 and 285 corresponding to the loss of two glucuronic acid moieties whereas compound **9** with an $[M-H]^-$ at *m/z* 623 gave fragment ions corresponding to the loss of a glucuronic acid and a glucose moiety. Based on comparison of UV and LC-MS data with those from the literature (Table 1), compounds **7** and **9** were tentatively identified as luteolin 7,4'-di-*O*-glucuronide and luteolin 7-*O*-glucuronide-3'-*O*-glucoside, respectively, which are compounds not previously found in *O. vulgare* subspecies.

Compounds **3** and **4** showed typical UV absorptions for flavanones with an intense band I centred around 285 nm and band II with a shoulder at around 320 nm, which was also confirmed by their LC-MS data showing an ion at *m/z* 287 corresponding to the aglycone eriodictyol. The MS spectrum of **3** with fragments corresponding to the ions E_1^-, E_2^-, $^{0,2}X^-$ and $^{0,3}X^-$, and the fragments $[M-H-240]^-$ and $[M-H-180]^-$ clearly suggested that this flavanone had a di-*C*-glucosyl substitution (Cuyckens et al. 2000; Waridel et al. 2001). Based on these observations, compound **3** was tentatively identified as eriodictyol 6,8-di-*C*-glucoside. Compound **4** was identified as eriodictyol 7-*O*-glucoside based on comparison of its UV and LC-MS data with an authentic standard. Eriodictyol and glycosidic eriodictyol derivatives have previously been isolated from Lamiaceae plant species (Parejo et al. 2004; Koukoulista et al. 2006a, b) but this is the first report of **3** and **4** in *O. vulgare* subspecies.

Recently, lithospermic acid B and its epimer *epi*-lithospermic acid B were isolated from the aerial parts of *O. vulgare* ssp. *hirtum* (Koukoulista et al. 2006a, b). The pseudomolecular ion $[M-H]^-$ at *m/z* 717 for compounds **18** and **19** together with their characteristic UV absorptions at 254 nm and a complex band with three absorption maximums at 286, 308 and 330 nm (Table 1) were consistent with **18** and **19** being *epi*-lithospermic acid B and lithospermic acid B, respectively. The identity of lithospermic acid B was confirmed by NMR analysis.

Yield of plant material, dry matter and volatile terpenes

The yield of *O. vulgare* ssp. *hirtum* plant material and dry matter (DM) content in the individual harvests are listed in Table 2 together with the yield of total and major volatile terpenes. The highest fresh weight (FW) yield of aerial parts was 12.7 t ha^{-1} in 2003 but only 6.1 t ha^{-1} in 2004. Berghold et al. (2008) reported a yield ranging from 8 to 16 t ha^{-1} for five *O. vulgare* strains cultivated in

Table 2. Effects of development stage at harvest on fresh weight (FW) yield of aerial parts, content of dry matter (DM), content of total volatile terpenes, yield of total volatile terpenes and the content of major volatile terpenes (carvacrol, thymol, *p*-cymene, γ-terpinene, myrcene, β-caryophyllene, α-pinene) in *Origanum vulgare* ssp. *hirtum*. The aerial parts of the oregano crops were harvested five or four times per year in two following years. The plots from 2nd harvest in 2003 were re-harvested once in autumn. Data given as means (n = 3).

Harvest date	Development stage relative to flowering	Biomass yield FW (t ha^{-1})	Biomass DM (%)	Volatile terpenes DM (%)	Total yield of volatile terpenes (kg ha^{-1})	Major volatile terpenes (mg g^{-1} DM)						
						carvacrol	thymol	*p*-cymene	γ-terpinene	myrcene	β-caryophyllene	α-pinene
2003												
2. July	5 % o. fl.	7.7 c*	22.2 a	4.3 ab	74.2 d	32.0 ab	0.4 b	2.1 c	4.3 a	1.1 ab	0.7 b	0.7 b
11. July	20 % o. fl.	8.5 c	25.4 b	4.2 ab	89.8 cd	31.5 ab	0.5 b	2.1 c	3.7 a	1.0 ab	0.7 b	0.7 b
23. July	50 % o. fl.	12.7 a	26.2 b	4.9 a	164.6 a	37.2 a	0.7 a	2.3 bc	3.9 a	1.2 a	0.9 a	0.9 a
6. Aug.	Full flowering	10.1 b	29.4 c	4.4 ab	131.5 ab	34.8 ab	0.6 a	2.9 a	2.3 b	0.9 b	0.7 b	0.7 b
15. Aug.	50 % s. fl.	10.1 b	32.6 d	3.7 b	122.1 bc	29.6 b	0.5 b	2.8 ab	1.5 bc	0.7 c	0.6 b	0.5 c
24. Oct.	Re-harvest, no o. fl.	2.0 d	35.8 e	1.6 c	11.9 e	10.3 c	0.2 c	3.0 a	1.0 c	0.4 d	0.2 c	0.3 d
2004												
12. July	10 % o. fl.	4.7 bc	25.1 c	2.6 b	31.2 c	19.3 c	0.1 c	1.5 c	3.2 b	0.5 c	0.2 b	0.4 c
26. July	30 % o. fl.	4.6 c	27.2 ab	4.0 a	49.5 b	27.3 b	0.2 b	2.9 b	6.2 a	0.9 b	0.4 a	0.6 b
11. Aug.	60 % o. fl.	6.1 a	28.6a	4.6 a	79.0 a	30.6 ab	0.2 ab	4.3 a	6.1 a	1.2 a	0.5 a	0.8 a
25. Aug.	10 % s. fl.	5.7 ab	26.2bc	4.5a	67.4 a	32.4 a	0.2 a	3.4 ab	5.3 a	1.2 a	0.5 a	0.9 a

*Means followed by the same letter in year groups are not significantly different due to separation by Duncan's multiple range test ($P < 0.05$).
Aug. = August, Oct. = October; o. = open, fl. = flowers, s. = senescent.

Austria with a relatively high content of essential oil (up to 4–5 % in DM). A high concentration of carvacrol (around 70 %) and low β-caryophyllene content (around 2 %) in the essential oil of these strains indicate that they belong to the subspecies *O. vulgare* ssp. *hirtum*. Röhricht and Mänicke (2004) reported *O. vulgare* ssp. *hirtum* yields of 2.5 t dry leaves ha^{-1} per year as a mean of six years of experiments in Germany (Location: Roda, Saxony). With an anticipated DM content of 20–25 % this would equal a FW yield of leaves around 10–12 t ha^{-1}.

In Denmark only one harvest of *O. vulgare* ssp. *hirtum* per year seems to be profitable (Table 2 re-harvest), whereas two to three harvests are possible in the Mediterranean region. Also Röhricht and Mänicke (2004) reported that in 4 out of 6 experimental years no re-harvest was possible in the Saxony area. Yields of total volatile terpenes (Table 2) varied from 75–165 kg ha^{-1} (2003) and 30–80 kg ha^{-1} (2004) being highest at near full flowering stage (50–60 % open flowers or 23 July 2003 and 11 August 2004). Berghold et al. (2008) harvested the highest yield of essential oil a little earlier in plant development (10 to 40 % open flowers) and the different strains yielded from about 50 to 160 kg oil ha^{-1} (56 to 180 L oil ha^{-1} density 0.9 kg L^{-1}) depending on year and *O. vulgare* strain.

The yield of volatile terpenes in *O. vulgare* ssp. *hirtum* plant material (Table 2) was much higher in 2003 than in 2004 and this was probably due to both locations and the better growing conditions in 2003. The summer (June–August) temperature in 2004 was on average 2 °C lower than in 2003 (17.4 °C *vs* 15.4 °C) and a re-harvest in autumn of 2004 was therefore not possible.

The concentrations of volatile terpenes were almost the same in both years with a range of 3.7–4.9 % (w/w based on DM) in 2003 (excl. re-harvest) and 2.6–4.6 % in 2004 (Table 2). The volatile terpene content was first slightly increasing with development stage until around 50 % open flowers and then decreasing again with later development (Table 2). Berghold et al. (2008) also found an overall increasing content of essential oil with later development stages of their plants (first cut only), but they only harvested until about 40 % open flowers. Russo et al. (1998) reported a similar amount of oil content (2.25–5.73 %) for 24 accessions of *O. vulgare* ssp. *hirtum* collected in southern Italy.

Content and composition of volatile terpenes

The major volatile terpenes identified in *O. vulgare* ssp. *hirtum* (Table 2 and 3) in 2003 and 2004 were carvacrol (75.8 %, 67.4 %), γ-terpinene (8.0 %, 13.5 %), *p*-cymene (4.7 %, 9.5 %), myrcene (2.5 %, 2.6 %), α-pinene (1.8 %, 1.9 %), β-caryophyllene (1.9 %, 1.0 %) and thymol (1.4 %, 0.4 %). The percentages in brackets are the fraction of a single volatile terpene of the total volatile mass analysed in the plant material from the third harvest

Table 3. Effects of development stage at harvest on the composition of the major volatile terpenes in *Origanum vulgare* ssp. *hirtum*. The 7 volatile terpenes represent over 95 % of the total volatile terpenes in the dry matter of aerial plant parts. Experimental details as in Table 2.

Harvest date	Development stage relative to flowering	Composition of major volatile terpenes (% w/w of total volatile terpenes)						
		carvacrol	thymol	*p*-cymene	γ-terpinene	myrcene	β-caryophyllene	α-pinene
2003								
2. July	5 % o. fl.	74 c*	1.0 bc	4.9 b	9.9 a	2.4 a	1.6 bc	1.6 bc
11. July	20 % o. fl.	76 bc	1.2 b	5.1 b	8.9 ab	2.4 ab	1.7 ab	1.6 bc
23. July	50 % o. fl.	76 bc	1.4 a	4.7 b	8.0 b	2.5 a	1.9 a	1.8 ab
6. Aug.	Full flowering	79 ab	1.5 a	6.6 b	5.2 c	2.1 b	1.7 ab	1.6 bc
15. Aug.	50 % s. fl.	80 a	1.4 a	7.4 b	4.2 c	1.8 c	1.7 ab	1.4 c
24. Oct.	Re-harvest, no o. fl.	64 d	0.9 c	18.2 a	6.2 c	2.3 ab	1.4 c	2.0 a
2004								
12. July	10 % o. fl.	74 a	0.4 a	5.6 b	12.4 a	2.0 b	0.7 b	1.4 b
26. July	30 % o. fl.	68 b	0.4 a	7.2 b	15.5 a	2.4 ab	1.0 a	1.6 ab
11. Aug.	60 % o. fl.	67 b	0.4 a	9.5 a	13.5 a	2.6 a	1.0 a	1.8 a
25. Aug.	10 % s. fl.	71 ab	0.4 a	7.5 ab	11.6 a	2.6 a	1.0 a	1.9 a

*Means followed by the same letter in year groups are not significantly different due to separation by Duncan's multiple range test ($P < 0.05$).
Aug. = August, Oct. = October; o. = open, fl. = flowers, s. = senescent.

Table 4. Effects of development stage at harvest on content (in mg g^{-1} DM) of total flavonoids, total phenolic acids and content of major flavonoids (eriodictyol 6,8-di-*C*-glucoside (**3**), apigenin 6,8-di-*C*-glucoside (**5**), and luteolin 7-*O*-glucuronide (**11**)) and major phenolic acids (rosmarinic acid (**16**), *epi*-Lithospermic acid B (**18**) and lithospermic acid B (**19**)) in *Origanum vulgare* ssp. *hirtum*. Experimental details as in Table 2.

Harvest date	Development stage relative to flowering	Total flavonoids	Major flavonoids			Total phenolic acids	Major phenolic acids		
			3	**5**	**11**		**16**	**18**	**19**
2003									
2 July	5 % o. fl.	5.4 bc*	0.9 a	0.7 d	0.7 a	8.7 a	1.2 ab	0.2 a	5.2 ab
11 July	20 % o. fl.	5.8 bc	0.8 ab	0.9 c	0.6 a	10.1 a	1.8 ab	0.2 a	6.2 a
23 July	50 % o. fl.	7.7 a	0.6 ab	1.2 b	0.5 ab	4.9 ab	1.0 ab	0.2 a	2.5 ab
6 Aug.	Full flowering	6.7 ab	0.5 bc	1.5 a	0.5 ab	4.4 ab	0.9 ab	0.1 a	2.4 ab
15 Aug.	50 % s. fl.	4.8 c	0.3 cd	1.5 a	0.2 b	1.4 b	0.3 a	0.1 a	0.7 b
24 Oct.	Re-harvest, no o. fl.	4.3 c	0.1 d	0.9 c	0.5 ab	9.1 a	2.2 b	0.2 a	5.6 a
2004									
12 July	10 % o. fl.	8.9 b	1.8 b	0.8 c	2.5 a	51.5 a	35.1 a	6.4 a	8.0 a
26 July	30 % o. fl.	12.2 a	2.8 a	1.4 b	2.4 a	40.5 b	26.3 b	5.6 a	7.0 a
11 Aug.	60 % o. fl.	12.4 a	1.8 b	2.2 a	1.8 b	38.7 b	26.2 b	5.0 ab	6.2 a
25 Aug.	10 % s. fl.	8.9 b	1.1 b	2.4 a	1.5 b	23.4 c	14.4 c	3.8 b	4.4 b

*Means followed by the same letter in year groups are not significantly different due to separation by Duncan's multiple range test ($P < 0.05$).
Aug. = August, Oct. = October; o. = open, fl. = flowers, s. = senescent.

time in year 2003 and 2004, respectively. Berghold et al. (2008) found a similar distribution of major compounds in the essential oil distilled from one of the strains of *O. vulgare* ssp. (strain 10) they investigated (carvacrol 74 %, γ-terpinene 7.3 %, *p*-cymene 3.4 %, myrcene 2.2 %, α-pinene 0.8 %, β-caryophyllene 1.6 % and thymol < 0.5 %) and confirmed that the composition is influenced by both biotic and abiotic circumstances during growth.

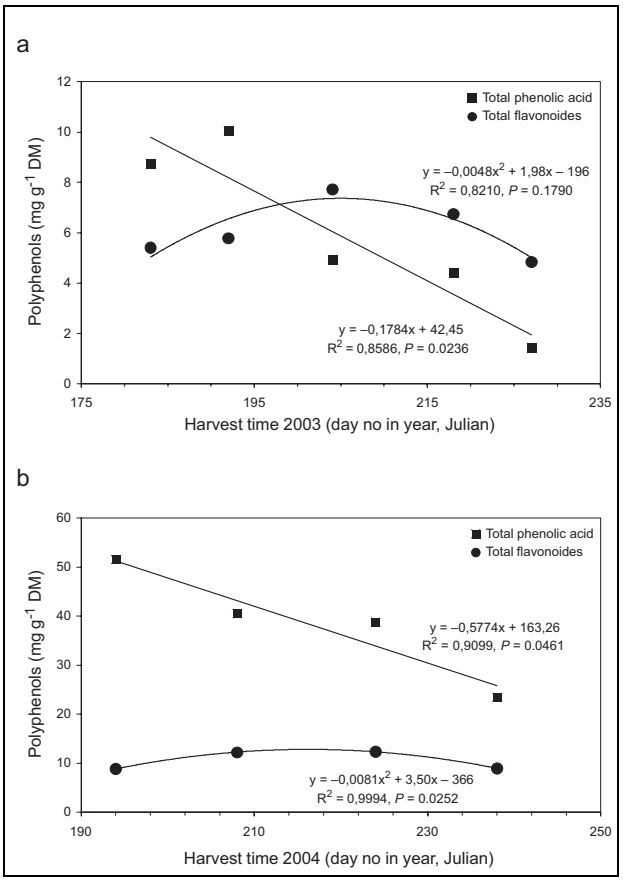


Fig. 5. Effects of harvest time as Julian day number in year on the concentration of total flavonoids and total phenolic acids in *Origanum vulgare* ssp. *hirtum* in year 2003 (a) and 2004 (b). Data are averages of three replicates per harvest. The parameters of a linear relationship for phenolic acids *vs* day number and a parabolic relationship for flavonoids *vs* day number are indicated as well as the R-square and P-values (n = 4 or 5).

Röhricht and Mänicke (2004) found an average of 81 % carvacrol in their *O vulgare* ssp. *hirtum* strains (range over years and strains 67 %–88 %) and about 0.5 % β-caryophyllene in accordance with the present results. Significant changes in concentration (Table 2) and composition (Table 3) of the different volatile terpenes were observed at the different development stages. The concentrations of the major volatile terpenes changed during the different development stages but the profile of volatile terpenes remains more or less unchanged. In particular, re-harvesting in October significantly affected the relative abundance of carvacrol (lower) and the abundance of its biosynthetic precursor's *p*-cymene (higher) compared to harvesting at the flowering stage in July (Fig. 3). Contradictory to this, Berghold et al. (2008) measured a relatively higher content of carvacrol (average about 2 % points higher) in the essential oil from the second cutting in most of their strains, which may be explained by the different development stages of the harvested plants. The second cutting in the present investigation was very late (24 October) and the plants were still in vegetative stage opposite to the plants in the experiment by Berghold et al. (2008). In 2004 there was a significantly higher relative content of γ-terpinene (12–16 %) and lower carvacrol content (67–74 %) compared to the crop harvested in 2003 (Table 3). The reason for this is not known but may again be due to differences in location and growing conditions. The results of Berghold et al. (2008) and Röhricht and Mänicke (2004) are based on distillation of essential oil components of dry plant material. Still their results are comparable to the present results obtained by extraction of essential components from fresh frozen plant material, although drying of plant material may result in small losses of essential oil components and distillation may result in small losses of essential oil components and artefact formation due to heating during the distillation process. Therefore, it is expected that the content and composition of volatile terpenes determined in the present investigation largely reflect the outcome after an industrial postharvest processing.

Content and composition of flavonoids and phenolic acids

The concentration of total flavonoids in *O. vulgare* ssp. *hirtum* was at its highest near full flowering, whereas the concentration of phenolic acids decreased steadily from first to last harvest. The magnitude of concentrations of total phenolic acids were about five times higher in 2004 than in 2003 (Table 4). Still the tendency with declining concentration with later harvest could be described by a significant linear correlation (Fig. 5) in both years (2003: R^2 = 0.8586, P = 0.0236* and 2004: R^2 = 0.9099, P = 0.0461*, respectively). The concentration of flavonoids relative to harvest time could be described by a parabolic function (2003: R^2 = 0.8210, P = 0.1790 (ns) and 2004: R^2 = 0.9994, P = 0.0252*, respectively), but this relationship was only significant in year 2004 (Fig. 5). Röhricht and Mänicke (2004) found an average of 1.5 % rosmarinic acid in their *O. vulgare* ssp. *hirtum* strains and about 0.4 % flavonoids. The content of rosmarinic acid in dry matter of our *O. vulgare* ssp. *hirtum* was only about 0.1–0.2 % in 2003 and about 1.4–3.5 % in 2004 (Table 4). This large variation can only be explained by differences in growing conditions in the two years. Röhricht and Mänicke (2004) also stated that there can be large variations in the content of phenolic compounds over years, but they, on the other hand, only found a magnitude of variation in rosmarinic acid content of about 7 times (0.6 % to 4.1 %). Because of the large differences in magnitude of the content of phenolic compounds over years, it is not possible to set up a general model of the potential yield of phenolic compound in relation to harvest time in *O. vulgare* ssp. *hirtum*.

Conclusion

The highest yield of both volatile terpenes and flavonoids in *O. vulgare* ssp. *hirtum* is obtained just before full flowering, whereas the highest yield of phenolic acids is obtained earlier around 20 % flowering. In the case of phenolic acids, a large variation in harvested plant material of *O. vulgare* ssp. *hirtum* must be expected. Therefore, in conclusion, the development stage has a significant impact on the content and composition of both terpenes and polyphenols in *O. vulgare* ssp. *hirtum*, and the optimal harvest time depends on the compounds of interest.

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Addresses of authors: Kai Grevsen (corresponding author), Department of Horticulture, Faculty of Agricultural Sciences, University of Aarhus, Kirstinebjergvej 10, DK-5792 Aarslev, Denmark, and Xavier C. Fretté and Lars P. Christensen, Institute of Chemical Engineering, Biotechnology and Environmental Technology, Faculty of Engineering, University of Southern Denmark, Niels Bohrs Allé 1, DK-5230 Odense M, Denmark, e-mail: kai.grevsen@agrsci.dk.