Polyketide synthases in Cannabis sativa L

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Chapter III

Polyketide synthase activities and biosynthesis of cannabinoids and flavonoids in *Cannabis sativa* L. plants.

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Abstract

Polyketide synthase (PKS) enzymatic activities were analyzed in crude protein extracts from cannabis plant tissues. Chalcone synthase (CHS, EC 2.3.1.74), stilbene synthase (STS, EC 2.3.1.95), phlorisovalerophenone synthase (VPS, EC 2.3.1.156), isobutyrophenone synthase (BUS) and olivetol synthase activities were detected during the development and growth of glandular trichomes on bracts. Cannabinoid biosynthesis and accumulation take place in these glandular trichomes. In the biosynthesis of the first precursor of cannabinoids, olivetolic acid, a PKS could be involved; however, no activity for an olivetolic acid-forming PKS was detected. Content analyses of cannabinoids and flavonoids, two secondary metabolites present in this plant, from plant tissues revealed differences in their distribution, suggesting a diverse regulatory control on these biosynthetic fluxes in the plant.

III.1 Introduction

Cannabis sativa L. is an annual dioecious plant from Central Asia. Cannabinoids are the best known group of natural products in *C. sativa* and 70 of these have been found so far (ElSohly and Slade, 2005). Several therapeutic effects of cannabinoids have been reported (reviewed in Williamson and Evans, 2000) and the discovery of an endocannabinoid system in mammalians marks a renewed interest in these compounds (Di Marzo and De Petrocellis, 2006; Di Marzo *et al*., 2007). The cannabinoid biosynthetic pathway has been partially elucidated (Figure 1). It is known that the geranyl diphosphate (GPP) and the olivetolic acid are initial precursors, which are derived from the deoxyxylulose phosphate/methyl-erythritol phosphate (DOXP/MEP) pathway (Fellermeier *et al*., 2001) and from the polyketide pathway (Shoyama *et al*., 1975), respectively. These precursors are condensed by the prenylase geranyl diphosphate:olivetolate geranyltransferase (Fellermeier and Zenk, 1998) to yield CBGA; which is further oxido-cyclized into CBDA, Δ^9-THCA and CBCA (Morimoto *et al*., 1999) by the enzymes cannabidiolic acid synthase (Taura *et al*., 2007b), Δ^9-tetrahydrocannabinolic acid synthase (Sirikantaramas *et al*., 2004) and cannabichromenic acid synthase (Morimoto *et al*., 1998), respectively. On the other hand, the first step leading to olivetolic acid, an alkylresorcinolic acid, is less known and it has been proposed that a polyketide synthase (PKS) could be involved in its biosynthesis. Raharjo *et al*. (2004a) found *in vitro* enzymatic activity for a PKS from leaves and flowers, though yielding olivetol and not the olivetolic acid as the reaction product. Olivetolic acid is the active form for the next biosynthetic reaction step of the cannabinoids. Later, a *PKS* mRNA was detected from leaves, which expressed activity for the PKSs chalcone synthase (CHS), phlorisovalerophenone synthase (VPS) and isobutyrophenone synthase (BUS), but not for the formation of olivetolic acid (Raharjo *et al*., 2004b).

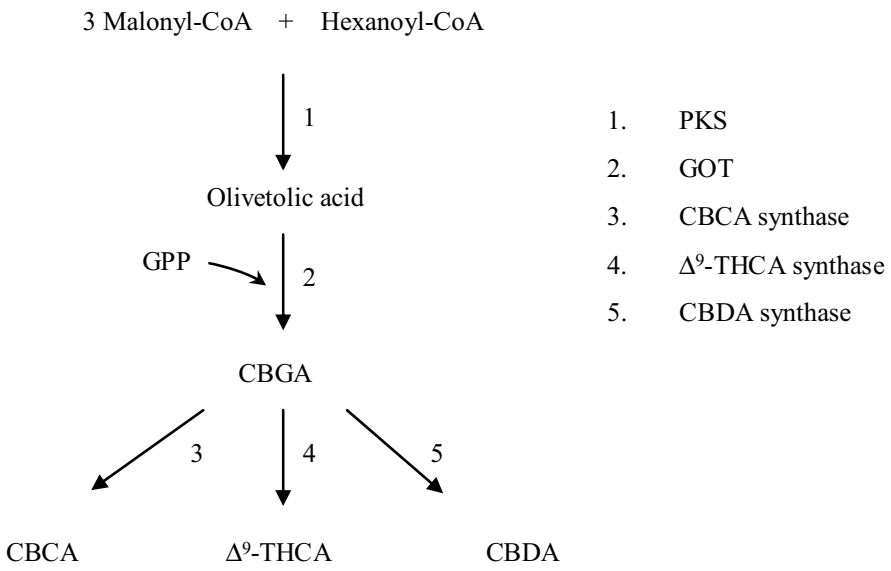


Figure 1. General pathway for biosynthesis of cannabinoids. PKS, polyketide synthase; GPP, geranyl diphosphate; GOT, geranyl diphosphate:olivetolate geranyltransferase; CBGA, cannabigerolic acid; Δ9-THCA , Δ9-Tetrahydrocannabinolic acid; CBDA, cannabidiolic acid; CBCA, cannabicromenic acid.

PKSs are a group of condensing enzymes that catalyzes the initial key reactions in the biosynthesis of a myriad of secondary metabolites (Schröder, 1997). In plants several PKSs have been found, which participate in the biosynthesis of compounds from the secondary metabolism. CHS, STS, VPS, BUS, bibenzyl synthase (BBS), homoeriodictyol/eriodictyol synthase (HEDS or HvCHS) and stilbene carboxylate synthase (STSC) are some examples from type III PKSs as they have been classified (Austin and Noel, 2003; Eckermann *et al.*, 2003; Klingauf *et al.*, 2005; Chapter II). Type III PKSs use a variety of thioesters of coenzyme A as substrates from aliphatic-CoA to aromatic-CoA, from small (acetyl-CoA) to bulky (*p*-coumaroyl-CoA) or from polar (malonyl-CoA) to nonpolar (isovaleryl-CoA). For example, CHS (Kreuzaler and Hahlbrock, 1972) and STS (Rupprich and Kindl, 1978) condense one molecule of *p*-coumaroyl-CoA with 3 molecules of malonyl-CoA forming naringenin-chalcone and resveratrol, respectively. VPS (Paniego *et al.*, 1999) and biphenyl synthase (Liu *et al.*, 2007) uses isovaleryl-CoA and benzoyl-CoA, respectively, as starter substrates instead of *p*-coumaroyl-CoA.

Here, we report the PKS enzymatic activities found in different tissues of cannabis plants and show a correlation between the production of polyketide derived secondary metabolites and the activity of these PKSs in the plant.

III.2 Materials and methods

III.2.1 Plant material

Seeds of *Cannabis sativa*, variety Skunk (The Sensi Seed Bank, Amsterdam, The Netherlands), were germinated and 9 day-old seedlings were planted in 11 LC pots with soil (substrate 45 L, Holland Potgrond, Van der Knaap Group, Kwintsheul, The Netherlands) and maintained under a light intensity of 1930 lux, at 26 °C and 60% relative humidity (RH). After 3 weeks the small plants were transplanted into 10 L pots for continued growth until flowering. To initiate flowering, 2 month-old plants were transferred to a photoperiod chamber (12 h light, 27 °C and 40% RH). Young leaves from 13 week-old plants, female flowers in different stages of development and male flowers from 4 month-old plants were harvested. Three month-old male plants were used for pollination of female plants. The fruits were harvested 18 days after pollination. Roots from 4 month-old female plants were harvested and washed with cold water to remove residual soil. All vegetal material was weighed and stored at -80 °C.

III.2.2 Chemicals

Benzoyl-CoA, hexanoyl-CoA, isobutyryl-CoA, isovaleryl-CoA, malonyl-CoA, resveratrol, naringenin and 2,4-dihydroxy-benzoic acid were obtained from Sigma (St. Louis, MO, USA). Olivetol was acquired from Aldrich Chem (Milwaukee, WI, USA) and 4-hydroxybenzyledeneacetone (PHBA) from Alfa Aesar (Karlsruhe, Germany). Orcinolic acid (orsellinic acid) was from AApin Chemicals Ltd (Abingdon, UK) and resorcinol (1,3-dihydroxy-benzene from Merck Schuchardt OHG (München, Germany). *p*-Coumaroyl-CoA was synthesized according to Stöckigt and Zenk (1975), and phlorisovalerophenone (PiVP) and phlorisobutyrophenone (PiBP) were previously synthesized in our laboratory (Fung *et al*., 1994). Olivetolic acid was obtained from hydrolysis of methyl olivetolate (Horper and Marner, 1996) and methyl olivetolate was a gift from Prof. Dr. J. Tappey (Virginia Military Institute, USA). The cannabinoids Δ^9-THCA,

CBGA, Δ^9-THC, Δ^8-THC, CBG, CBD and CBN were isolated from plant materials previously in our laboratory (Hazekamp *et al.*, 2004). Δ^9-THVA was identified based on its relative retention time and UV spectra (Hazekamp *et al.*, 2005) and its quantification was relative to Δ^9-THCA. The flavonoids kaempferol, orientin and luteolin were purchased from Extrasynthese (Genay, France), and vitexin, isovitexin and apigenin from Sigma-Aldrich (Buchs, Switzerland). Quercetin, apigenin-7-*O*-Glc and luteolin-7-*O*-Glc were from our standard collection. All chemical products and mineral salts were of analytical grade.

III.2.3 Protein extracts

Frozen plant material was homogenized in a mortar with nitrogen liquid, the powder was thawed in polyvinylpolypyrrolidone (PVPP) and extraction buffer (0.1 M potassium phosphate buffer, pH 7, 0.5 M sucrose, 3 mM EDTA, 10 mM DTT and 0.1 mM leupeptin), squeezed through Miracloth and centrifuged at 14,000 rpm for 20 min. Per each gram of fresh weight, 0.1 g of PVPP and 2 ml of extraction buffer were used. The crude protein extracts were desalted using Sephadex G-25 M (PD-10) columns, eluted with same extraction buffer without addition of leupeptin. All steps were performed at 4 °C.

III.2.4 Polyketide synthase assays

Polyketide synthase activity was measured by the conversion of starter CoA esters and malonyl-CoA into reaction products.

The standard reaction mixture, in a final volume of 500 µl, contained 50 mM K-Pi buffer (pH 7), 20 µM starter-CoA, 40 µM malonyl-CoA 0.5 M sucrose and 1 mM DTT. The reaction was initiated by addition of 250 µl of desalted crude protein extracts (100-440 µg of protein) and was incubated for 90 min at 30 °C. Reactions were stopped by addition of 20 µl of 4N HCl then extracted twice with 800 µl of ethyl acetate and centrifuged for 2 min. The combined organic phases were evaporated in vacuum centrifuge and the residue was kept at 4 °C. Samples were resuspended in 100 µl and in 40 µl MeOH for analysis by HPLC and LC/MS, respectively.

VPS was isolated previously in our laboratory (Paniego *et al.*, 1999), and CHS and STS were a gift from Prof. Dr. J. Schröder (Freiburg University, Germany).

III.2.5 Protein determination

Protein concentration was measured as described by Peterson (1977) with bovine serum albumin as standard.

III.2.6 HPLC analysis

The system consisted of a Waters 626 pump, a Waters 600S controller, a Waters 2996 photodiode array detector and a Waters 717 plus autosampler (Waters, Milford, MA, USA), equipped with a reversed-phase C18 column (250 x 4.6 mm, Inertsil ODS-3, GL Sciences, Tokyo, Japan). 80 µl of sample was injected, the gradient solvent system consisted of MeOH and Water, both containing 0.1% TFA: Method 1) 0-40 min, 20-80% MeOH; 40-43 min, 80% MeOH,; 43-48 min, 80-20% MeOH; 40-50 min, 20% MeOH. Method 2) 0-30 min, 40-60% MeOH; 30-33 min, 60% MeOH; 35-38 min, 60-40% MeOH; 38-40 min 40% MeOH. Method 3) 0-40 min, 40-60% MeOH; 40-43 min, 60% MeOH; 43-44 min, 40-60% MeOH; 44-45 min 40% MeOH. Method 4) 0-40 min, 50-100% MeOH; 40-43 min, 100% MeOH; 43-44 min, 100-50% MeOH; 44-45 min, 50% MeOH. Method 5) 0-20min, 50-80% MeOH; 20-30min, 80% MeOH; 30-35 min, 80-50% MeOH; 35-40 min, 50% MeOH. Flow rate was 1 ml/min at 25 °C; olivetol, methyl olivetolate, olivetolic acid, PiVP, PiBP, naringenin and resveratrol were detected at 280 nm, orcinolic acid at 260 nm, orcinol at 273 nm and 2,4-dihydroxy-benzoic acid at 256 nm. PHBA was detected at 320 nm. Calibration curves with the respective standards were made.

III.2.7 LC-MS analysis

For the confirmation of the identity of enzymatic products, 20 µl of samples were analyzed in an Agilent 1100 Series LC/MS system (Agilent Technologies, Palo Alto, CA, USA) with positive/negative atmospheric pressure chemical ionization (APCI), using elution system method 5 with a flow rate of 0.5 ml/min. The optimum APCI conditions included a N_2 nebulizer pressure of 45 psi, a vaporizer temperature of 400 °C, a N_2 drying gas temperature of 350 °C at 10 L/min, a capillary voltage of 4000 V, a corona current of 4 µA, and a fragmentor voltage of 100 V. A reversed-phase C18 column (150 x4.6 mm, 5 µm, Zorbax Eclipse XDB-C18, Agilent) was used.

III.2.8 Extraction of compounds

Extraction was carried out as described by Choi *et al*. (2004) with slight modifications. To 0.1 g of lyophilized and ground plant material was added 4 ml MeOH:H_2O (1:1, v/v) and 4 ml $CHCl_3$, vortexed for 30 s and sonicated for 10 min. The mixtures were centrifuged in cold at 3000 rpm for 20 min. The MeOH:H_2O and $CHCl_3$ fractions were separated and evaporated. The extraction was performed twice. The extracts were resuspended on 1 ml of MeOH:H_2O (1:1) and $CHCl_3$, respectively; for the subsequent cannabinoid and flavonoid analyses.

III.2.9 Cannabinoid analysis by HPLC

The column used was a Grace Vydac (WR Grace, Columbia, MD, USA) C_{18} (250x4.6 mm MASS SPEC 218MS54, 5 μm) with a Waters Bondapak C_{18} guard column (2x20 mm, 50 μm). The solvent system and the operational conditions were the same as previously reported by Hazekamp *et al*. (2004). For preparation of samples, 100 μl of the $CHCl_3$ fraction from extraction was evaporated using N_2 gas. The samples were dissolved in 1 ml of EtOH and 20 μl was injected in the HPLC system. Cannabinoids were detected at 228 nm. Calibration curves with their respective standards were made.

III.2.11 Flavonoid analysis by HPLC

A reversed-phase C18 column (250 x4.6 mm, Inertsil ODS-3) was used. The solvent system and the operational conditions were as described by Justesen *et al*. (1998) with slight modifications. The mobile phase consisted of MeOH:Water (30:70, v/v) with 0.1% TFA (A) and MeOH with 0.1% TFA (B). The gradient was 25-86% B in 40 min followed by 86% B for 5 min and a gradient step from 86-25% B for 5 min at a flow-rate of 1 ml/min and at 25 °C. Twenty μl of resuspended hydrolyzed samples was injected. Retention times for aglycones were as follows: apigenin 23.02 min, kaempferol 21.95 min, luteolin 18.37 min, quercetin 16.37 min, isovitexin 5.32 min, vitexin 4.71 min and orientin 3.64 min; and for apigenin-7-*O*-Glc 10.7 min and luteolin-7-*O*-Glc 7.42 min. Flavones and flavonols were detected at their maximal UV absorbance (quercetin, 255 nm; kaempferol, 265.8 nm; apigenin, isovitexin and apigenin-7-*O*-Glc, 270 nm; and orientin, luteolin and luteolin-7-*O*-Glc, 350 nm). Flow rate was 1 ml/min at 25 °C. Calibration curves with their respective standards

were made. The standards apigenin and vitexin were dissolved in MeOH:DMSO (7:3), orientin in MeOH:DMSO (8:2, v/v), apigenin-7-*O*-Glc and luteolin-7-*O*-Glc in MeOH:DMSO (9:1, v/v); the rest of them only in MeOH.
The optimum APCI conditions for LC-MS analyses were as described above.

III.2.12 Acid hydrolysis for flavonoids
Five hundred microliters of the MeOH:H_2O fraction from extraction were hydrolyzed at 90 °C for 60 min with 500 µl of 4N HCl to which 2 mg of antioxidant *tert*-butylhydroquinone (TBHQ) was added. Hydrolysates were extracted with EtOAc three times. The organic phase was dried over anhydrous $NaSO_4$ and evaporated with N_2 gas.

III.2.13 Statistics
All data were analyzed by MultiExperiment Viewer MEV 4.0 software (Saeed *et al.*, 2003; Dana-Faber Cancer Institute, MA, USA). For analyses involving two and three or more groups paired *t*-test and ANOVA were used, respectively with $\alpha = 0.05$ for significance.

III.3 Results and discussion

III.3.1 Activities of PKSs present in plant tissues from *Cannabis sativa*

For positive control of PKS activity, CHS from *Pinus sylvestris*, STS from *Arachis hypogaea* and VPS from *Humulus lupulus* were used (Table 1). The activities of these enzymes were similar to the ones previously reported of STS (58.6 pKat/mg protein) from peanut cell cultures (Schoppner and Kindl., 1984), CHS (30 pKat/mg protein) from *Phaseolus vulgaris* cell cultures (Whitehead and Dixon., 1983) and VPS (35.76 pKat/mg protein) from hop (Okada *et al.*, 2000), respectively. Negative control assays consisted on standard reaction mixture adding 50 µl water as starter and extender substrate. The final pH for CHS and benzalacetone synthase (BAS) assays was 8, which is optimum for the naringenin (Schröder *et al.*, 1979; Whitehead and Dixon, 1983) and benzalacetone (Abe *et al.*, 2001; Abe *et al.*, 2007) formation, while for the rest of PKS assays was maintained at 7. Due to limited availability of substrates and standards, for detection of STS type activity in cannabis protein extracts we decided to perform the assay using the starter substrate *p*-coumaroyl-CoA for

resveratrol formation as general indicator from STS activities. For detection of CHS type activities, the assay was carried out with *p*-coumaroyl-CoA as starter substrate and naringenin-chalcone formation was an indicator of CHS type activity. For detection of VPS and BUS activities, the assays were achieved with the starter substrates isovaleryl-CoA and isobutyryl-CoA, respectively.

Table 1. PKSs activities used as positive control. The enzymatic assays were made in a final reaction volume of 400 µl with 100 µl of purified enzyme (35-66 µg of protein).

PKS	Sp Act (pKat/mg protein)	Product
CHS (*Pinus sylvestris*)	33.30 ± 3.45	Naringenin
STS (*A. hypogaea*)	70.50 ± 5.02	Resveratrol
VPS (*H. lupulus*)	31.97 ± 6.86	Forming PiVP
VPS (*H. lupulus*)	27.66 ± 14.83	Forming PiBP

For the analysis of the assays of PKS activities by HPLC, we started with the eluent system reported by Robert *et al*. (2001), which was slightly modified as is described in material and methods (method 1). Narigenin (Rt 33.55 min) and resveratrol (Rt 26.36 min) had a good separation in this solvent system; however, the retention times of olivetol, PiVP and PiBP (Table 2) were longer than naringenin. Four elution gradients were tested in order to reduce the retention times of these standards and the method 5 was used subsequently for the analysis by HPLC and LC-MS.

Table 2. Retention times (min) of standards employed for analyses using a elution system of MeOH:H_2O in different gradient profiles.

Standard	Naringenin	Resveratrol	PiVP	PiBP	PHBA	Olivetol	Olivetolic acid	Methyl olivetolate	Orcinolic acid	Orcinol	2,4-dBZ acid	Resorcinol
Solvent system* 1	33.55	26.36	37.85	33.71	24.71	37.97	-	-	-	-	-	-
Solvent system* 2	24.69	13.65	33.95	26.09	10.88	33.22	-	-	-	-	-	-
Solvent system* 3	30.21	15.96	39.80	31.68	12.75	40.10	-	-	-	-	-	-
Solvent system* 4	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	-	-	-	-	-	-
Solvent system* 5	14.50	9.01	18.50	15.32	8.54	18.37	23.45	26.83	9.07	5.53	7.15	4.36

*see material and methods
2,4-dihydroxy-benzoic acid, 2,4-dBZ acid
n.r., no resolution
-, not measured

CHS activity was detected in the plant tissues analyzed (Figure 2) and maximum activities were observed in roots (24.86 ± 4.38 pKat/mg protein). No significant differences were found in the CHS activity from the rest of the tissues analyzed ($P<0.05$) which were until 16 times lesser than that one in roots. STS type activities were also detected in the same plant tissues. The STS activities from fruits and male leaves were no significant different (0.96 ± 0.07 pKat/mg protein and 1.05 ± 0.04 pKat/mg protein, respectively) as well as those ones from female leaves and male flowers (2.11 ± 0.12 pKat/mg protein and 1.76 ± 0.12 pKat/mg protein, respectively). The STS activities from bracts, seedlings and roots were 5 times higher than that one in fruits and they were not significant different. No VPS activities were detected in fruits and roots. The VPS activity in seedlings was until 15 times lesser than those in bracts and male flowers, which were not significantly different. The VPS activities detected in leaves (female and male) were until 7 times bigger than that one in seedlings (0.39 ± 0.06 pKat/mg protein) and they were not significant different by gender. Significant differences were observed in BUS activities from bracts, seedlings and leaves. The BUS activities from female leaves (7.98 ± 2.98 pKat/mg protein) and male leaves (5.76 ± 2.5 pKat/mg protein) were highly significant; no BUS activity was found in fruits, roots and male flowers.

PKS activities expressed during the development of the glandular trichomes on the bracts were significantly different ($P<0.05$), except at day 31(Figure 3). CHS activity was increased at day 23 during the growth and development of glandular hairs. The CHS activities at days 17 and 35 were not significantly different to the BUS and VPS activities at the same days. No significant differences were found in STS-type activity during the time course, except at day 35 when it had increased three fold. VPS and BUS activities increased during the growth and development of the glandular trichomes on female flowers with a maximum activity at day 23 (7.07 ± 1.05 pKat/mg protein) and 29 (15.99 ± 4.5 pKat/mg protein), respectively. During the accumulation of resin the VPS activities were not significantly different (from days 31 to 35), but BUS activities were significantly different during the time course. The activities from days 17, 29 and 31 were significantly different between BUS and VPS. No activity for an olivetolic acid-forming PKS was detected during the time course of the growth and development of glandular trichomes on female flowers. However, HPLC and LC-MS analyses confirmed formation of olivetol (retention time 18.21 ± 0.24

min and *m/z* 181 $[M+H]^+$) using hexanoyl-CoA as starter substrate. This PKS activity forming olivetol was not detected in seedlings, fruits and roots; but significant differences were found in bracts, male flowers and between the leaves of the two genders (Figure 2). The activity for this olivetol-forming PKS was seven times higher in bracts than that in male leaves (5.35 ± 1.07 pKat/mg protein). A time-course of the growth and development of glandular trichomes on female flowers showed that the activity of the olivetol-forming PKS increased at day 29 and decreased later until no activity was detected anymore in female flowers from 35 days-old (Figure 3). Raharjo *et al*. (2004a) suggested that olivetol was formed by a PKS and Kozubek and Tyman (1999) proposed that alkylresorcinols, such as olivetol, are formed from biosynthesized alkylresorcinolic acids by enzymatic decarboxylation or via modified fatty acid-synthesizing enzymes, where the olivetolic acid carboxylic group would be expected to be also attached either to ACP (acyl carrier protein) or to CoA. Thus, in the release of the molecule from the protein compartment in which it was attached or elongated, simultaneous decarboxylation of the olivetol may occur, otherwise the olivetolic acid would be the final product. PKS isolation and gene identification forming alkylresorcinolic acids (Gaucher and Shepherd, 1968; Gaisser *et al*., 1997; Funa *et al*., 2007) and stilbene carboxylic acids (Eckermann *et al*., 2003; Schröder Group) has been reported. Conversion of tetraketides (free acids or lactones) synthesized *in vivo* by stilbene carboxylic acid synthases (Schröder Group) or by chemical synthesis (Money *et al*., 1967) into the carboxylic acids at a suitable pH (mildly acidic or basic conditions) has been suggested too.

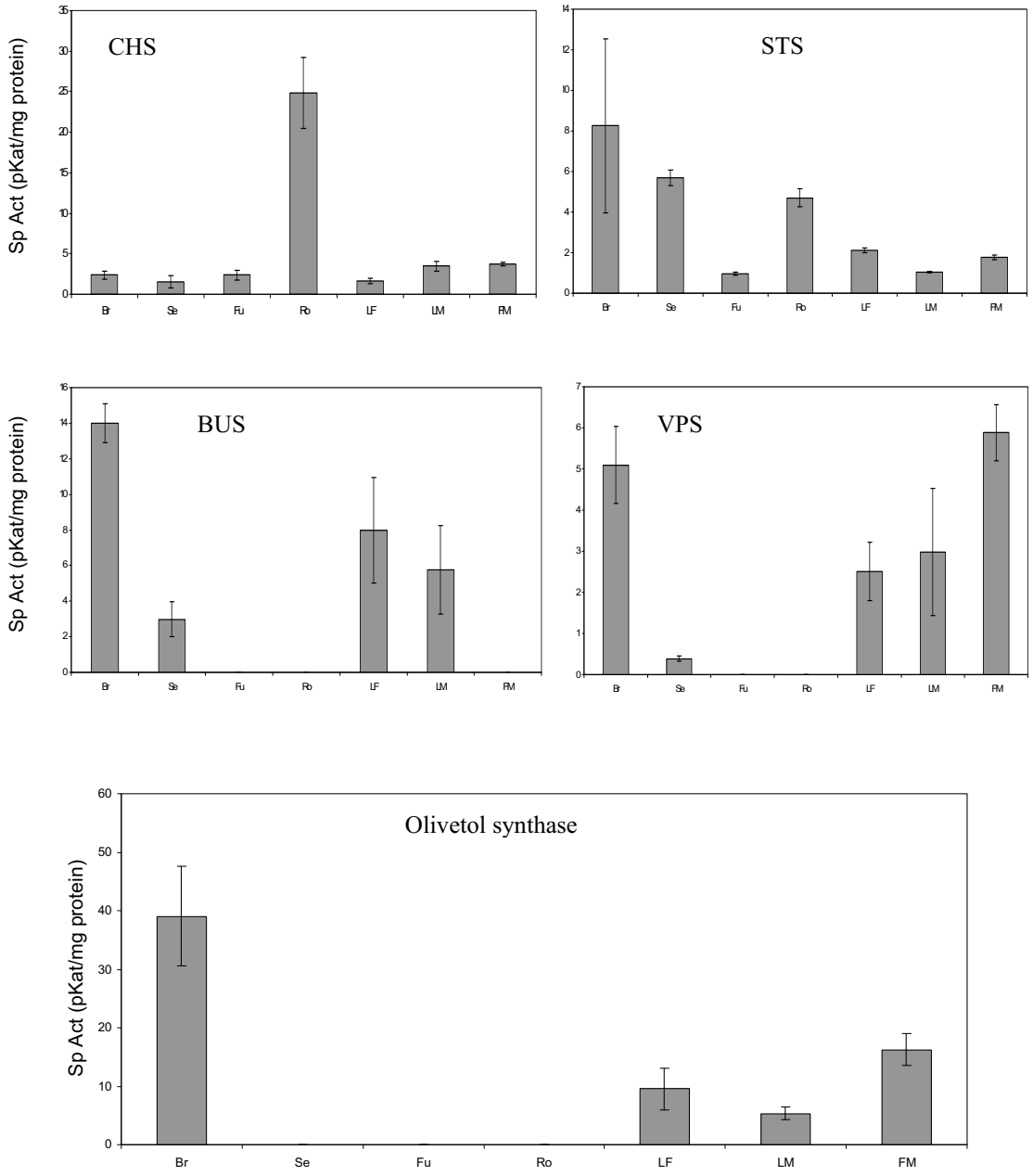


Figure 2. PKS activities in several crude extracts from different cannabis tissues. Br, bracts; Se, seedlings; Fu, fruits; Ro, roots; LF, female leaf; LM, male leaf; FM, male flower. Bracts of flowers from 29 day-old. Values are expressed as means of three replicates with standard deviations.

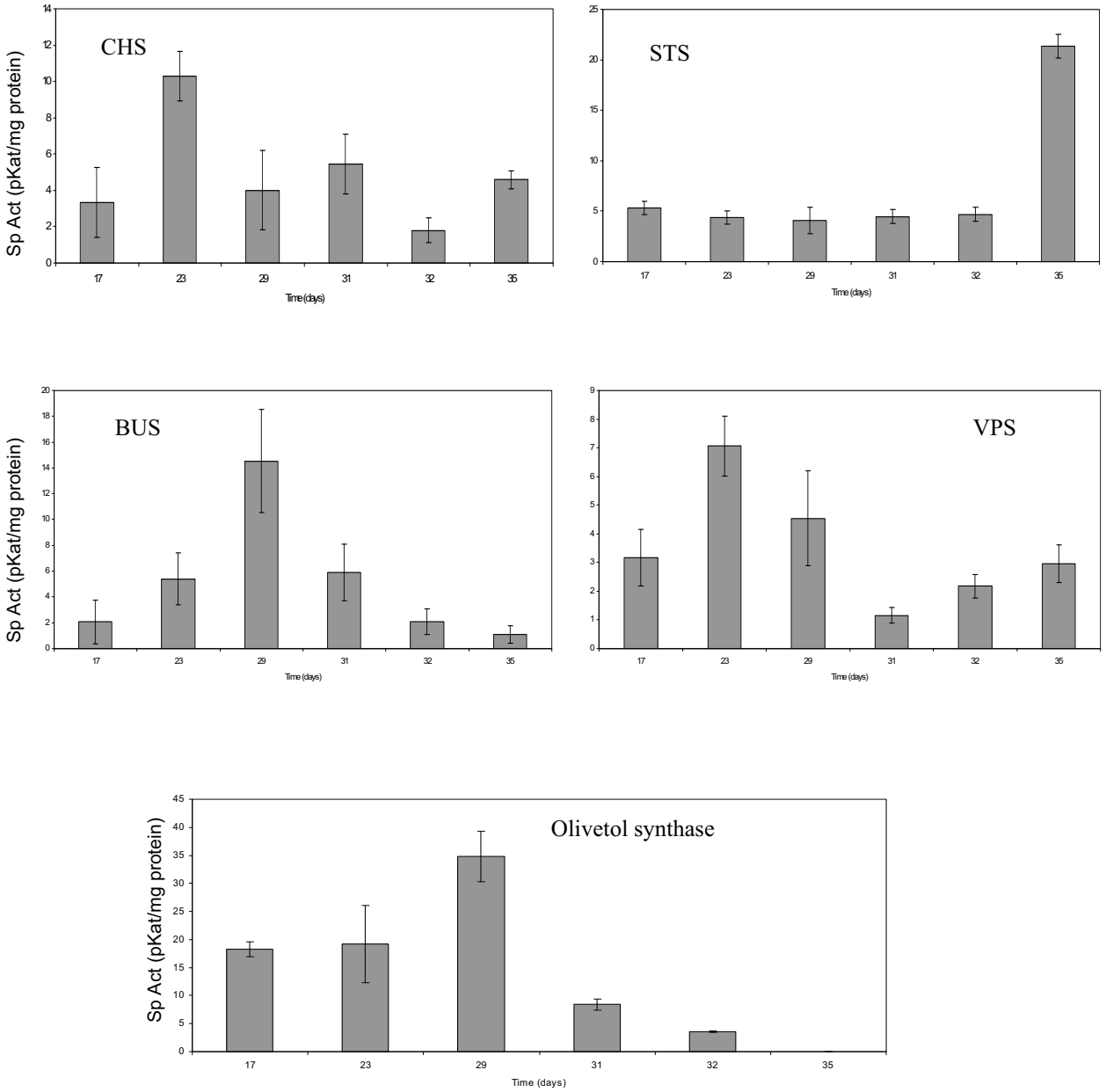


Figure 3. PKS activities during the development of glandular trichomes on female flowers. Values are expressed as means of three replicates with standard deviations.

Table 3. Recovery of olivetolic acid, orcinolic acid, 2,4-dihydroxy-benzoic acid (2,4-dBZ acid) and methyl-olivetolate from cannabis protein crude extracts.

Tissue	Standard	Addition	Added concentration	Calculated concentration	Recovery (%)
Bracts:					
	Olivetolic acid	After Rx	0.5 mg	0.48 ± 0.02	96.00
			60 µM	57.52 ± 2.18	95.87
		Before Rx	120 µM	116.31 ± 4.72	96.92
(C-)			120 µM	117.81 ± 0.54	98.18
			60 µM	58.76 ± 0.94	97.93
(C-)			60 µM	56.67 ± 1.66	94.45
	Orcinolic acid	After Rx	0.06 mg	0.058 ± 0.004	96.67
			60 µM	57.94 ± 1.51	96.57
		Before Rx	120 µM	117.31 ± 2.13	97.76
(C-)			120 µM	118.00 ± 1.41	98.33
			60 µM	59.42 ± 0.64	99.03
(C-)			60 µM	58.53 ± 2.05	97.55
	2,4-dBz acid	After Rx	0.01 mg	0.0098 ± 0.0002	98.00
			60 µM	57.26 ± 1.03	95.43
		Before Rx	120 µM	118.19 ± 1.86	98.50
(C-)			120 µM	118.87 ± 0.13	99.06
			60 µM	57.69 ± 3.09	96.15
(C-)			60 µM	57.31 ± 1.07	95.52
	Methyl-olivetolate	After Rx	0.5 mg	0.49 ± 0.017	98.00
			60 µM	58.87 ± 1.23	98.12
		Before Rx	120 µM	118.43 ± 1.89	98.69
(C-)			120 µM	119.47 ± 0.68	99.56
			60 µM	56.59 ± 2.59	94.32
(C-)			60 µM	58.41 ± 0.53	97.35
Leaves:					
	Olivetolic acid	Before Rx	20 µM	19.15 ± 0.01	95.75
	Orcinolic acid	Before Rx	20 µM	19.27 ± 0.92	96.35
	2,4-dBz acid	Before Rx	20 µM	19.24 ± 0.31	96.20

(C-), negative controls without addition of protein extract

Raharjo *et al*. (2004a) did not observe any effect on the formation of the olivetol by neither the incubation time of the PKS assays nor the mildly acidic conditions used. Enzymatic decarboxylation *in vitro* and *in vivo*, and purification of carboxylic acid decarboxylases has been reported from liverworts (Pryce, 1972; Pryce and Linton, 1974), lichens (Mosbach and Ehrensvard, 1966) and microorganism (Pettersson, 1965; Huang *et al*., 1994; Dhar *et al*., 2007; Stratford *et al*., 2007). We did not observe formation of olivetol by an enzymatic or chemical decarboxylation from olivetolic acid (Table 3). Although, the

recovery for the standards orcinolic acid and 2,4-dihydroxy-benzoic acid was more than 95% no orcinol or resorcinol (1,3-dihydroxy-benzene) was detected; methyl-olivetolate was used as negative control of decarboxylation. Purification of this olivetol-forming PKS is required in order to characterize it and analyze the mechanism of the reaction. In addition, no activity was detected with benzoyl-CoA at pH 7.0, 7.5 or 8.0 and no BAS activity was found. Slightly small amounts of derailment byproducts were detected from the PKS assays.

III.3.2 Cannabinoid profiling by HPLC

Figure 4 shows the variations in the cannabinoid content with respect to tissues analyzed. Eight times higher concentration of Δ^9-THCA was detected in female flowers than in male flowers. No significant differences were found in the contents in male flowers, fruits and male or female leaves ($P<0.05$). Previous studies confirm that there is no significant difference in the cannabinoid content in leaves of the two genders from the same variety (Holley *et al.*, 1975; Kushima *et al.*, 1980). Δ^9-THVA was only identified in male and female flowers, and fruits. The concentration of this cannabinoid in flowers was more than seven times higher than the content in fruits but the Δ^9-THVA content from male flowers was not significantly different from fruits. The CBGA contents from female flowers and, male and female leaves were not significantly different. The content of this cannabinoid in fruits was six times lesser than in female flowers. The CBGA concentration detected from male flowers was not significantly different from fruits. CBDA was identified in flowers and leaves; the CBDA content from female flowers was 2.6 times higher than in male flowers. The CBDA contents from leaves were not significantly different from male flowers. The increment of the concentration of cannabinoids corresponds with the development and growth of the glandular trichomes on the bracts (Table 4 and Figure 5). No significant differences were found in the CBGA and CBDA contents. Although cannabinoid content in the individual glandular trichomes can vary with age, type and location (Turner *et al.*, 1977; Turner *et al.*, 1978), a correlation exists between glandular density and cannabinoid content at each stage of bract development (Turner *et al.*, 1981). As CBGA is the precursor of Δ^9-THCA and CBDA, its concentration slightly decreased (from 0.18 ± 0.087 mg/100 mg dry weight to 0.12 ± 0.099 mg/100 mg dry weight). Δ^9-THCA content increased 1.6 times at day 31 (7.82 ± 2 mg/100 mg dry weight). On the

other hand, Δ^9-THVA accumulation started only after day 24. Natural (plant decarboxylation) or artificial degradation (oxidation, isomerization, UV-light) of cannabinoids occurred on lesser extent in our plant material (Table 4). No cannabinoids and neutral forms were found in seedlings and roots.

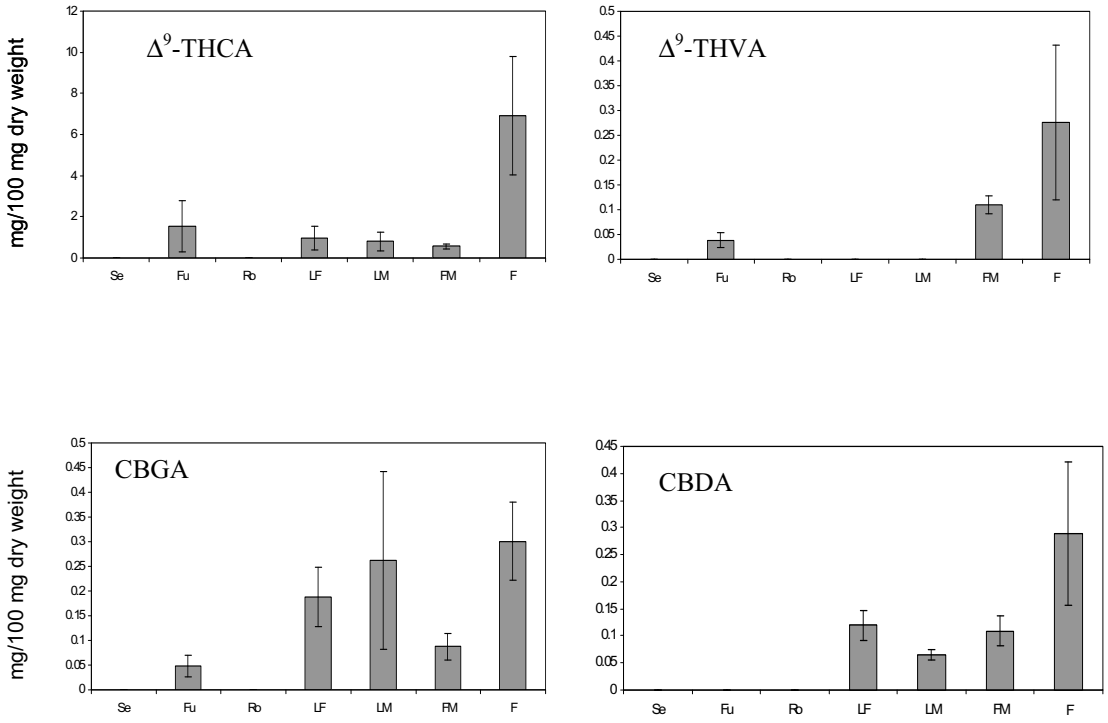


Figure 4. Cannabinoid content in different cannabis plant tissues. Br, bracts; Se, seedlings; Fu, fruits; Ro, roots; LF, female leaf; LM, male leaf; FM, male flower; F, female flower. Female flowers from 35 day-old. Values are expressed as means of three replicates with standard deviations.

Table 4. Cannabinoid content from different cannabis tissues.

Tissue	cannabinoids* (acid forms)	Δ^9-THC	CBG	CBD	CBN	Total
Bracts:						
24 d	5.19	0.42 ± 0.03	-	-	-	5.61
31 d	8.40	0.12 ± 0.03	-	-	0.08 ± 0.002	8.60
Fruits	1.63	0.04 ± 0.02	-	-	-	1.67
Leaves:						
Female	1.27	0.43 ± 0.31	-	-	0.06 ± 0.010	1.76
Male	1.13	-	-	-	-	1.13
Flowers:						
Female	7.78	0.16 ± 0.01	-	-	0.09 ± 0.005	8.03
Male	0.86	-	-	-	-	0.86

* concentration expressed in mg/100 mg dry weight; (Δ^9-THCA, Δ^9-THVA, CBDA and CBGA)
d, day

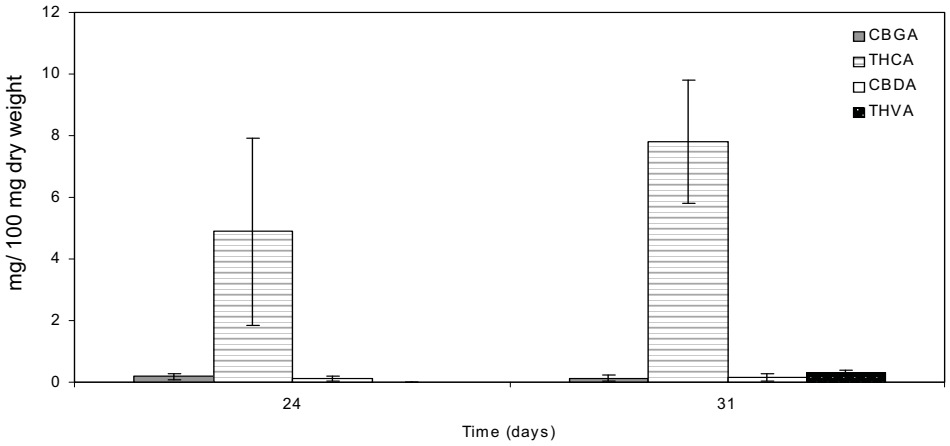


Figure 5. Cannabinoid content in bracts during the growth and development of glandular trichomes on female flowers.

III.3.4 Flavonoid profiling by HPLC

As standards for most flavonoid glycosides are not commercially available, we proceeded to hydrolyze the samples in order to analyze the aglycones. Apigenin, luteolin, apigenin-7-*O*-Glc and luteolin-7-*O*-Glc were used as internal standards. Percentage of recovery of aglycones from standards was more than 90% (Table 5). Typical profiles corresponding to a standard mixture of the selected flavones and flavonols with our samples are shown in figure 6 and analyses by LC-MS confirmed the identity of the aglycones (Figure 7).

Table 5. Recovery percentage of aglycones from standard acid hydrolysis.

Name	Concentration (mg)	Calculated concentration (mg)	% Recovery
Apigenin-7-*O*-Glc	0.3	0.283 ± 0.011	94
Apigenin	0.3	0.244 ± 0.012	81
Luteolin-7- *O*-Glc	0.3	0.277 ± 0.021	92
Luteolin	0.3	0.246 ± 0.019	82

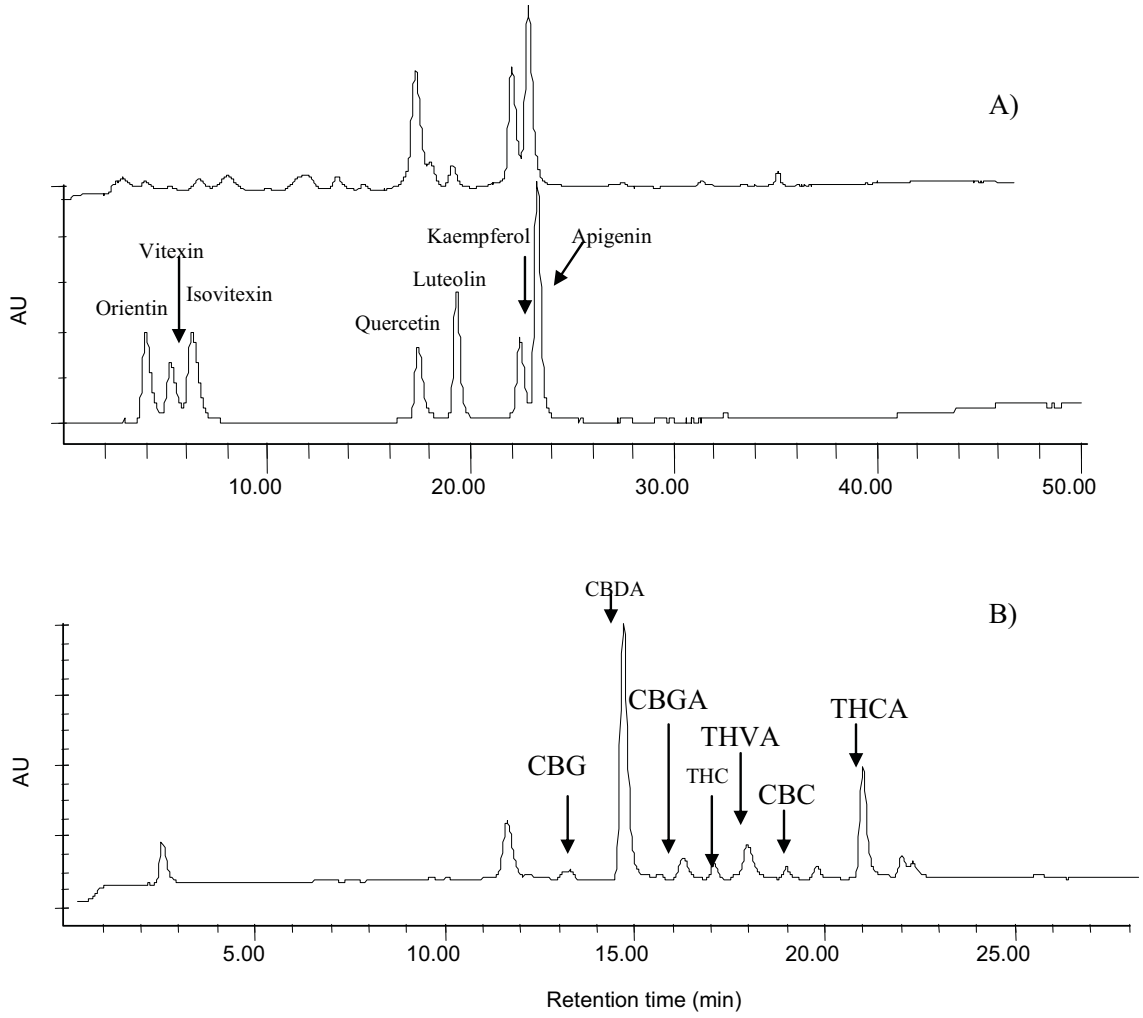
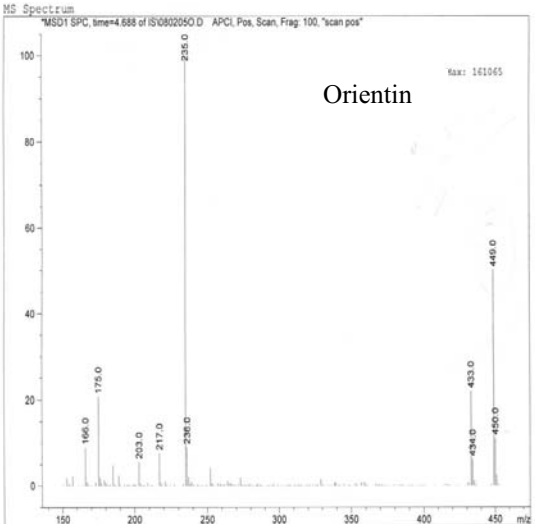
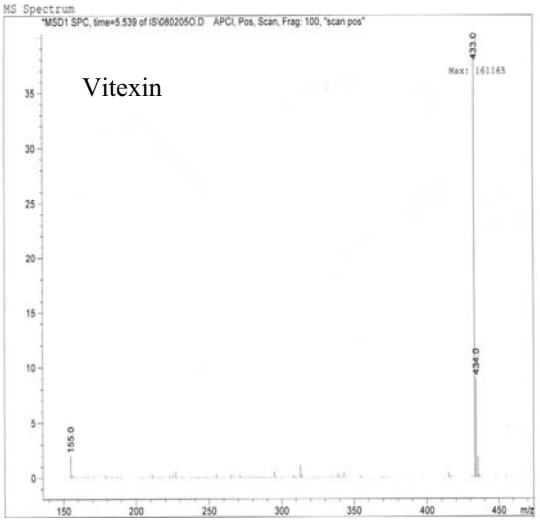
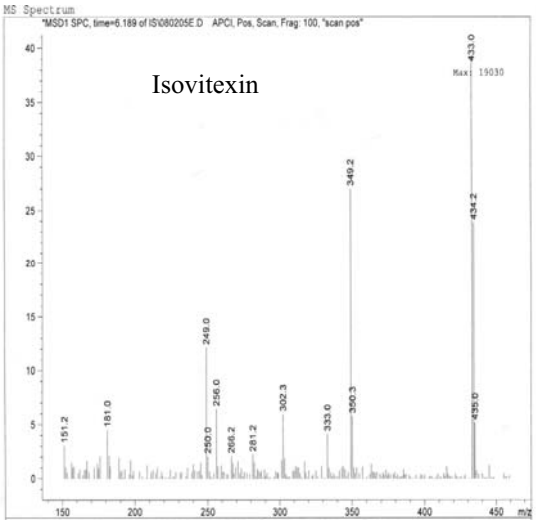
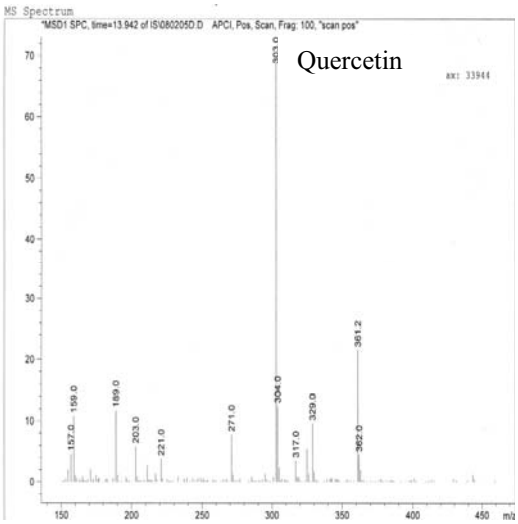


Figure 6. A) Comparison of HPLC chromatograms of the standard mixture of aglycones and a hydrolyzed MeOH:Water fraction (350 nm) and B) HPLC chromatogram of the chloroform fraction from bracts variety "Kompolti" (280 nm).


MS Spectrum
"MSD1 SPC, time=4.688 of IS\080205O.D APCI, Pos, Scan, Frag: 100, "scan pos"
Max: 161065
Orientin
235.0
449.0
175.0
433.0
166.0
203.0
217.0
236.0
434.0
450.0
m/z


MS Spectrum
"MSD1 SPC, time=5.539 of IS\080205O.D APCI, Pos, Scan, Frag: 100, "scan pos"
Vitexin
433.0
Max: 161165
434.0
155.0
m/z


MS Spectrum
"MSD1 SPC, time=6.189 of IS\080205E.D APCI, Pos, Scan, Frag: 100, "scan pos"
Isovitexin
433.0
Max: 19030
349.2
434.2
249.0
256.0
151.2
181.0
250.0
266.2
281.2
302.3
333.0
350.3
455.0
m/z


MS Spectrum
"MSD1 SPC, time=13.942 of IS\080205D.D APCI, Pos, Scan, Frag: 100, "scan pos"
Quercetin
303.0
361.2
304.0
159.0
189.0
157.0
203.0
271.0
221.0
329.0
317.0
362.0
m/z

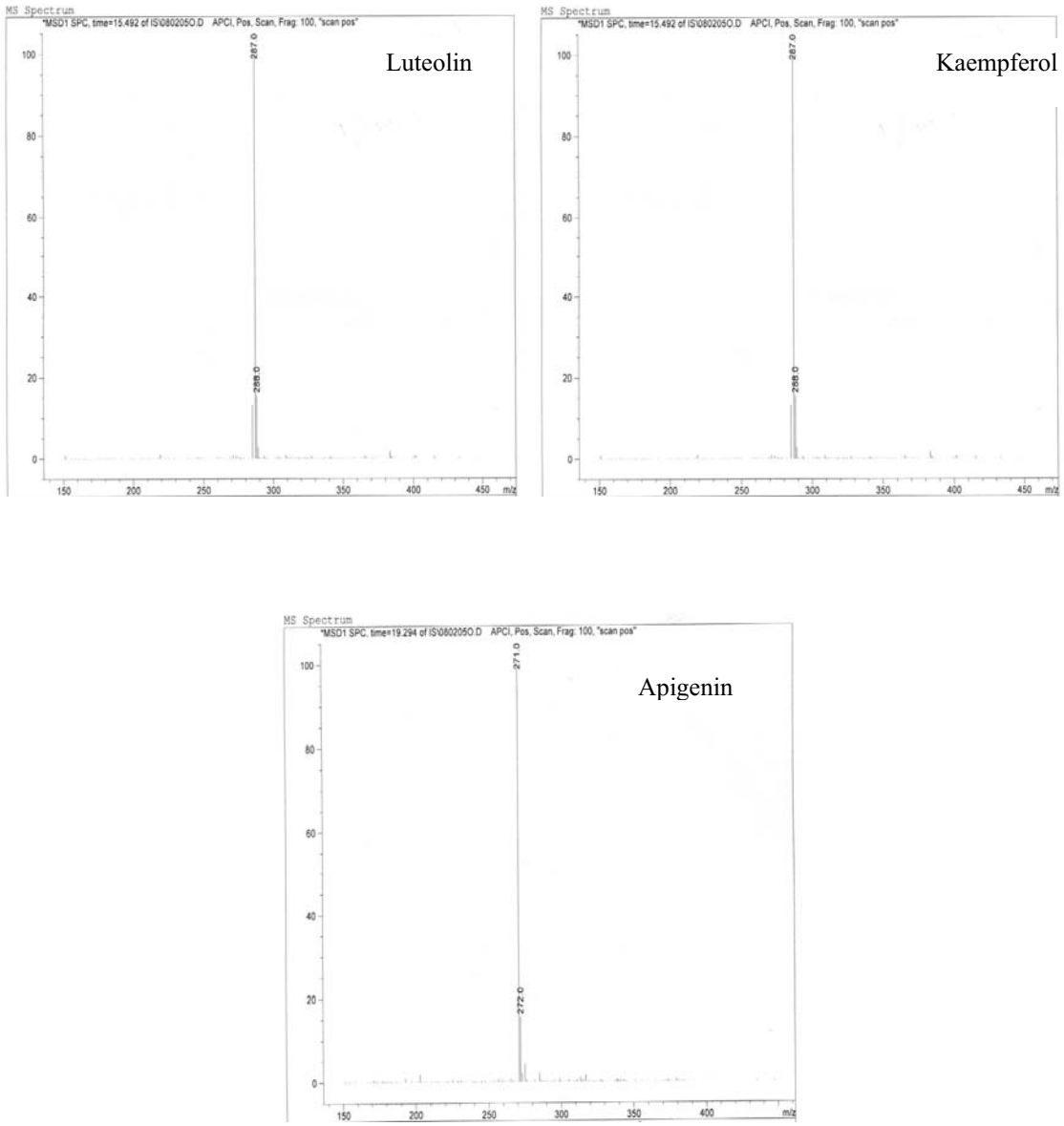


Figure 7. Mass-spectra of hydrolyzed flavonoids from MeOH:Water fraction in the range of m/z 150-450 obtained by LC-MS. Peak values correspond to [M+H]. *MW*: orientin, 448.4; vitexin, 432.4, isovitexin, 432.4; quercetin, 302.25; luteolin, 286.25; kaempferol, 286.25 and apigenin, 270.25.

Flavonoid content varied from a plant tissue to another (Figure 8). No flavonoids were detected in roots. Orientin content in flowers and leaves was not significant different by gender, but a significant difference was found between the contents from seedlings (0.040 ± 0.025 mg/100 mg dry weight) and fruits 0.026 ± 0.019 mg/100 mg dry weight). Vitexin content in fruits was the lowest and the contents in leaves and flowers were not significantly different. Isovitexin contents from female and male leaves were not significantly different, as well as the contents in seedlings and female flowers, and fruits and male flowers. Lowest amounts of quercetin were detected in fruits and highest amounts in male flowers. No significant differences were found in the contents in leaves and seedlings. The contents of luteolin in leaves (female and male), male flowers and seedlings were not significantly different and lowest contents were detected in fruits, which were not significantly different from the contents in male flowers. The kaempferol contents of leaves (female and male) and male flowers were not significantly different. Lowest contents were detected in fruits (0.0025 ± 0.0013 mg/100 mg dry weight) and the contents in seedlings and female flowers were seventeen times higher than in fruits. Apigenin contents from leaves were not significantly different for gender, but the contents in flowers were significantly different for gender. Lowest contents were detected in fruits (0.0048 ± 0.0028 mg/100 mg dry weight). Luteolin and vitexin contents are similar to results reported by Vanhoenacker *et al*., (2002) but apigenin and orientin contents are higher in our samples. Though Raharjo (2004) only reported apigenin and luteolin in leaves and flowers of *C. sativa* Fourway plants the contents were different from our results, probably because of differences in plant tissue age and the variety. Contrary to the cannabinoid accumulation during the growth and development of glandular trichomes the flavonoid content decreased (Figure 9 and Table 6).

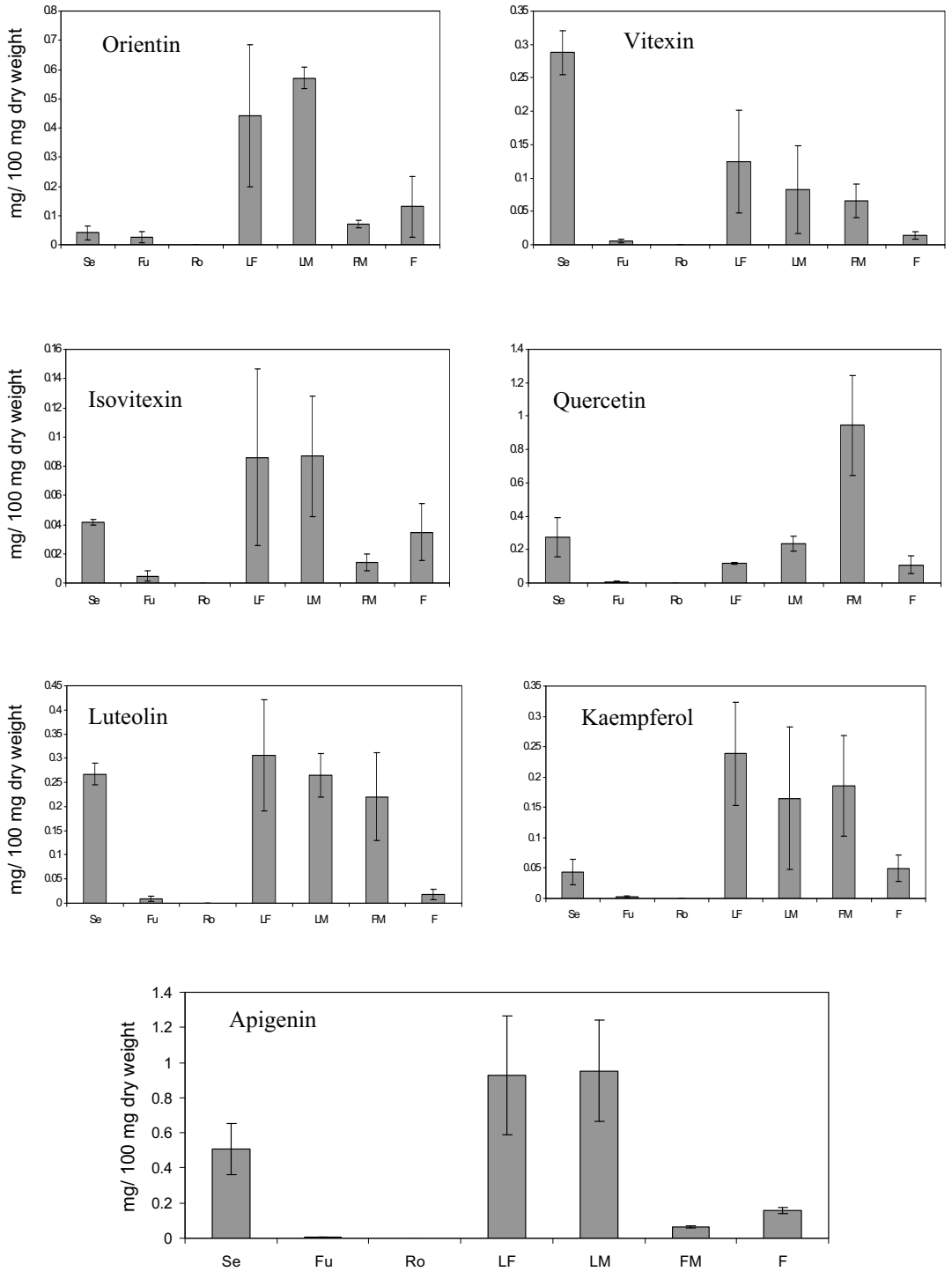


Figure 8. Flavonoid content in different cannabis plant tissues. Se, seedlings; Fu, fruits; Ro, roots; LF, female leaf; F, female flower; LM, male leaf; FM, male flower. Female flowers from 35 days-old. Values are expressed as means of three replicates with standard deviations.

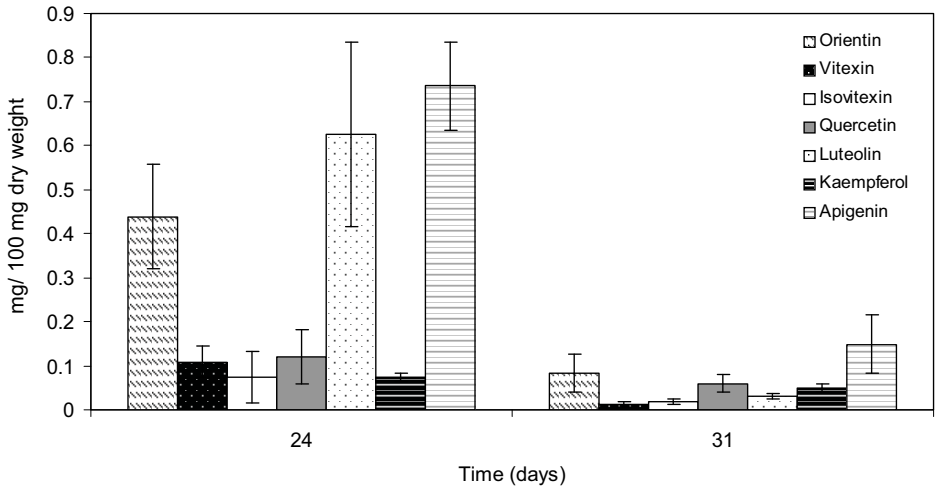


Figure 9. Flavonoid content in bracts during the growth and development of glandular trichomes on female flowers.

Table 6. Flavonoid content in different plant tissues from *C. sativa*.

Tissue	Flavonoid total content (mg/100 mg dry weight)
Bracts:	
24 d	2.18
31 d	0.40
Fruits	0.06
Seedlings	1.46
Leaves:	
Female	2.24
Male	2.36
Flowers:	
Female	1.56
Male	0.51

d, day

III.3.5 PKS activities and secondary metabolites in *C. sativa*

In plant tissues from *C. sativa*, *in vitro* PKS activities of CHS, STS, BUS and VPS, as well as activity for an olivetol-forming PKS were detected. Content analyses of cannabinoids and flavonoids, two secondary metabolites present in this plant (Chapter 1), revealed differences in their distribution, suggesting a diverse regulatory control on the biosynthetic fluxes in the plant. Apigenin, luteolin, kaempferol are widespread compounds in plants (Valant-Vetschera and Wollenweber, 2006). Quercetin and kaempferol have a role in fertility of male flowers (Vogt *et al.*, 1995; Napoli *et al.*, 1999) and higher levels of these two flavonols in cannabis male flowers than in female flowers (Figure 8) support this role. UV-B (280-315 nm) protection by flavone or flavonol glycosides has been reported (Lois and Buchanan, 1994; Rozema *et al.*, 2002) and their occurrence in aerial tissues from cannabis should be vital. Furthermore, roles as growth regulators have been suggested (Ylstra *et al.*, 1994; Gould and Lister, 2006). Quercetin, apigenin and kaempferol can modulate auxin-mediated processes (Jacobs and Rubery, 1988) and this role should not be excluded in cannabis. It has been reported that luteolin and apigenin derivatives acted as feeding deterrents of Lepidoptera larvae (Erhard *et al.*, 2007). On the other hand, it is known that cannabinoids are cytotoxic compounds (Rothschild *et al.*, 1977; Roy and Dutta, 2003; Sirikantaramas *et al.*, 2005) and they can act as plant defense compounds against predators such as insects. Moreover, a regulatory role in cell death has been suggested as cannabinoids have the ability to induce cell death through mitochondrial permeability transition (Morimoto *et al.*, 2007).

The accumulation of cannabinoids in bracts during the growth and development of glandular trichomes from flowers (Figure 5) could be related to floral protection and consequently during the seed maturation the cannabinoid content may decrease. Lower contents of cannabinoids were detected in fruits (seed and cup-like bracteole) than in female flowers (Table 3). It seems that cannabinoid accumulation is correlated with maximum activities for an olivetol-forming PKS (Figures 3 and 5) and the CHS activity preceded the accumulation of flavonoids at day 24 (Figures 3 and 9). A significant STS-type activity was detected at day 35 (Figure 3). Although, significant enzymatic activities for VPS and BUS were also detected in crude protein extracts no acylphloroglucinols have been identified in cannabis so far (Chapter I). Acylphloroglucinols and activities of VPS and BUS have been detected in *Humulus lupulus* (Paniego *et al.*, 1999) and *Hypericum perforatum* (Hoelzl and Petersen, 2003; Klingauf *et al.*, 2005). It is known that PKSs can use efficiently a broad range of substrates (Novak *et al.*, 2006; Springob *et al.*, 2000; Samappito *et al.*, 2003; Chapter II) and probably the cannabis PKSs have this notorious *in vitro* substrate promiscuity. Zuurbier *et al.* (1998) showed that CHS and STS enzymes can have VPS- and BUS-type activities and the VPS and BUS activities identified in this study could be from CHS or olivetol-forming PKS, even from STS. Although, a significant activity of CHS and STS activities were detected in crude protein extracts from roots (Figures 2) no flavonoids were identified in these tissues (Figure 8). There are no reports about isolation or detection of flavonoids and stilbenoids in roots (Chapter I) and contradict the CHS- and STS-type activities detected in roots. Low expression of the *CHS-type PKS* gene in roots and the absence of flavonoids in this plant tissue was previously reported (Raharjo *et al.*, 2004b; Raharjo 2004). Stilbenoids have been isolated from cannabis leaves and resin (Chapter I) but they could not be identified in the methanol:water fractions from leaves and bracts by LC-MS analysis, this could be due to the low STS-type activity (Figures 3). Gehlert and Kindl (1991) found a relationship between induced formation by wounding of stilbenes and the PKS BBS in orchids. Stilbenoid functions in plants include constitutive and inducible defense mechanisms (Chiron *et al.*, 2001; Jeandet *et al.*, 2002), plant growth inhibitors and dormancy factors (Gorham, 1980).

It is known that induction of enzymatic activity in early steps from a biosynthetic pathway precedes the accumulation of final products (Figure 10).

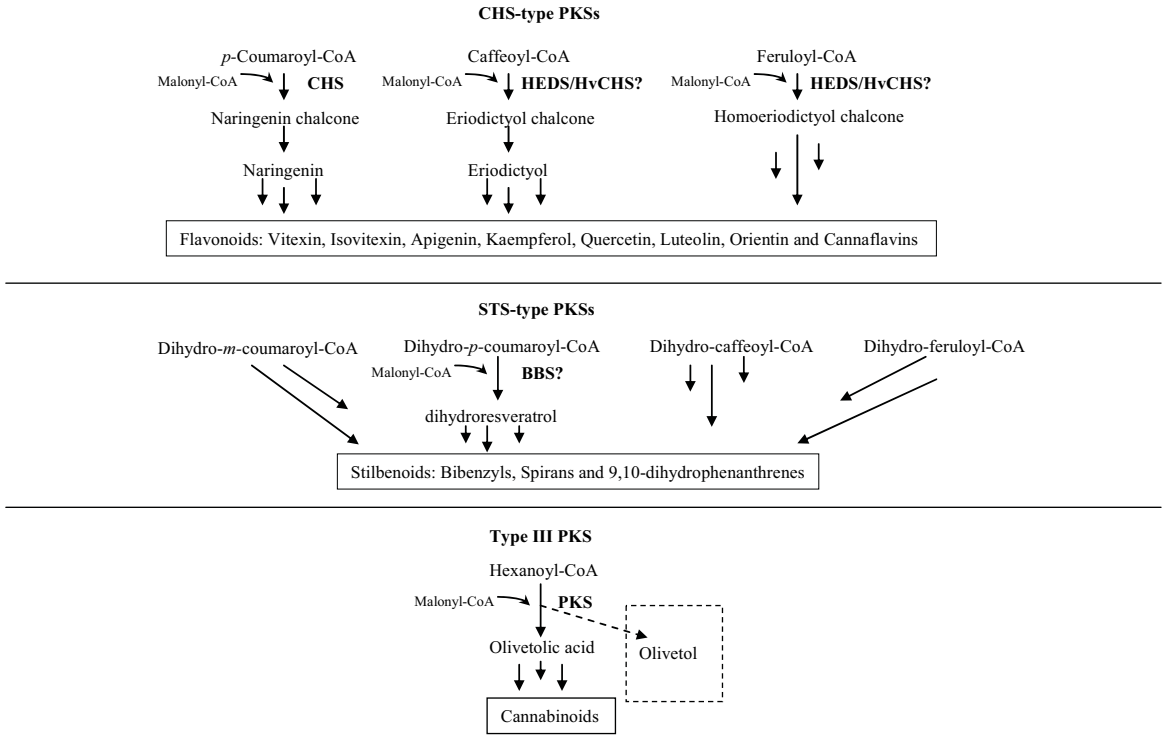


Figure 10. Proposed reactions for PKSs in the biosynthesis of precursors from flavonoid, stilbenoid and cannabinoid pathways in cannabis plants. Dashed square represent the compound found in crude extracts.

The cannabinoid content in female flowers was 5 times higher than the flavonoid content (Table 4) and during the development of the glandular trichomes on the flowers the activity of the olivetol-forming PKS at day 29 was 8 times higher than the CHS activity (Figure 3). Although, STS activity detected during the time course was low it increased at the end being 4 times and 21 times higher than the CHS and olivetol-forming PKS, respectively. This STS activity can be associated to the precursor formation in stilbenoid biosynthesis. The results shown here suggest the presence of three PKS activities, one CHS type, one STS type and another for the olivetol biosynthesis. However, further studies are required to identify the substrate specificities of these PKSs in cannabis plants. Purification and characterization of the PKS enzymes will be necessary to know their catalytic potential and their regulation, which may lead to the identification of their role in the plant.

Acknowledgements

We thank A. Hazekamp for the technical assistance on the flavonoid and cannabinoid analyses by LC-MS and HPLC and A. Garza Ortiz for the technical assistance on me-olivetolate hydrolysis. I.J. Flores Sanchez received a partial grant from CONACYT (Mexico).