Chapter III - Phytochemical study of *Coreopsis tinctoria* flowering tops

3.1 Flavonoid structural analysis – Analytical methods

A herbal product contains multiple constituents that might be responsible for its therapeutic effects. It is thus necessary to define as many constituents as possible in order to understand and explain the bioactivity. It is essential to elaborate the herbal product's chemical profile.

In earlier times flavonoids and phenolics in general, were only separated using techniques such as thin-layer chromatography (TLC), polyamide chromatography, and paper electrophoresis. Nowadays TLC is still widely used during fractionation work, for preliminary screening of plant extracts, essentially due to its simplicity, rapidity, versatility and low cost. However the majority of recently published papers refer to high performance liquid chromatography (HPLC) analysis (Boros et al., 2010; Inbaraj et al., 2010; Plazonic et al., 2009; Olsen, et al., 2009). Flavonoids can be separated, identified and quantified in one operation by coupling HPLC to Ultraviolet (UV), mass (MS), or nuclear magnetic resonance (NMR) detectors (Marston and Hostettman, 2006).

Flavonoid separation is usually accomplished through HPLC using octadecylsilyl bonded phases (ODS, RP-18 or C_{18}) and binary mobile phases composed by acidified water and polar organic solvents like acetonitrile or methanol. Acid modifiers are necessary to suppress the ionization of phenolic hydroxyl groups, giving sharper peaks with less tailing (Tsao and Deng, 2004; Wang and Huang, 2004). Flavonoid glycosides are eluted before aglycones with these phases, and flavonoids possessing more hydroxyl groups are eluted before the less substituted analogs (Croizier, A. et al., 1997).

In general, HPLC coupled with diode array and mass spectrometric detection provides an efficient method for rapid identification of flavonoids in a mixture, once it allows structure elucidation of the compounds separated by HPLC.

One particular feature that greatly aids flavonoid analysis, is the presence of an UV active chromophore: the phenyl ring. This not only makes flavonoids easy to detect but provides important structural information that can distinguish the oxidation pattern and type of polyphenol (Marston and Hostettman, 2006). All flavonoid aglycones contain at least two aromatic rings and, consequently, efficiently absorb UV light. The flavonoids in methanolic solutions typically yield two UV absorption peaks: The first maximum, which is found in the 240–285 nm range (band I), is due to the A-ring – and the second maximum, which is in the 300–400 nm range (band II), to the substitution pattern and conjugation of the B-ring (Markham and Mabry, 1975; Mabry et al., 1970).

When analysing flavonoids by MS with ESI it is common to observe the loss of small neutral molecules (such as: H_2O, CO, CO_2), radicals ($\dot{C}H_3$ in methoxylated compounds), parts of the molecule (glycoside unit). Cleavages in ring C results from Retro-Diels-Alder reactions which are very informative about substitution patterns in rings A and B (Cuyckens and Claeys, 2004). Information about carbohydrate sequence and the aglycone moiety can also be obtained through mass spectrometric methods; Cleavage at the glycosidic O-linkages with a concomitant H-rearrangement leads to the elimination of monosaccharide residues such as the loss of 162 a.m.u. (hexose) (Cuyckens and Claeys, 2004). Negative ionization mode is excellent for flavonoid analysis (Wang and Huang, 2004). Compared to the positive-ion ESI mode, negative-ion MS spectral characteristics are relatively simple with strong $[M-H]^-$ ions and very low abundance of fragment ions corresponding to sequential loss of sugar moieties. Lower fragmentation results in greater molecular ion abundances, and lower chemical noise with consequent higher signal-to-noise ratios for the molecular analyte species and hence greater sensitivity (Farag et al., 2007).

Tandem mass spectrometry (MS/MS) is used to produce structural information about a compound by fragmenting specific sample ions inside the mass spectrometer and identifying the resulting fragment ions. It also enables specific compounds to be detected in complex mixtures on account of their specific and characteristic fragmentation patterns.

3.2. Isolation and identification of main compounds

Coreopsis tinctoria has been traditionally used in Portugal as an antidiabetic and although there are already some studies on the phytochemistry of *C. tinctoria* flowering tops (Chapter II) little has been done to chemically characterize the bioactive extracts.

Hence, the aim of this chapter was to elaborate the phytochemical profile of the bioactive extracts and identify the main compounds involved in the corresponding antidiabetic activity. Simultaneously *in vivo* experiments were elaborated (Chapter 4) which allowed determining whether an extract/fraction was active or not.

The chosen approach was based on the ethnopharmacological data available. Therefore extraction followed the traditional preparation (an infusion) described by D'Oliveira Feijão in 1973 (5 g flowering tops in 1 cup of boiled water). The resulting aqueous extract (plant material ***CTD1***; sample A) presented an average yield of 30% (w/w dry plant material) and was the starting point from which a series of liquid-liquid fractionations with hexane:ethyl acetate (Hex:AcOEt 50%) and ethyl acetate (AcOEt 100%) followed.

Fractionation follow-up was achieved using TLC. A summary of the spots present in the extract and fractions, their Rf (Retention factor) and appearance after NP/PEG reagent spraying, are registered in Table 3.1.

Table 3.1. Spot appearance, Rf, colour after revelation and presence in the aqueous extract and subsequent fractions: Hex:AcOEt 50% and AcOEt 100%. Chromatographic system: silica gel/ C_6H_6:AcOEt:HCOOH (9:7:3).

Rf	Spot colour after NP/PEG		Presence in extracts/fractions		
	Visible	UV 366 nm	Aqueous Extract	Hex:AcOEt 50% Fraction	AcOEt 100% Fraction
0.05	Brown	Orange	+	-	++
0.1	Purple	Red	+++	-	++++
0.15	Yellow	Yellow/Orange	+	-	++
0.25	Pink	Yellow/Green	+	-	++
0.4	-	Green	+	+	++
0.75	Pink	Orange	++	+++	+
0.85	Yellow	Yellow/White	+	++	-
0.95	Pink	Pink	+	++	-

Legend: (-) Absent; (+) to (++++) Presence and spot intensity

C. tinctoria aqueous extract has clearly shown fluorescent spots at both 254 and 366 nm after TLC plate spraying with NP/PEG reagent and thus contain phenolic compounds.

The compound in spot of Rf = 0.1 seems to be the most abundant one in the aqueous extract of *C. tinctoria* as it presents an intense purple spot after being sprayed with NP/PEG reagent.

Yellow/Orange and Yellow/Green fluorescence after spraying with NP/PEG (Table 3.1) is diagnostic of flavones, flavonols and flavanones (Wagner and Bladt, 2003) thus there is an elevated prevalence of this type of phenolic compounds in *C. tinctoria* aqueous extract and subsequent fractions.

When comparing the TLC chromatografic profile of aqueous extract, Hex:AcOEt 50% and AcOEt 100% fractions, it can be observed that less polar compounds (which migrated more on the TLC-silica plate) have been successively separated from those with medium to high polarity that have been found in the subsequent 100% AcOEt fraction.

3.2.1. Structural identification of compound CT-1: Flavanokanin (88)

As described in the experimental section (Chapter 8) fractionation of 341 mg of Hex:AcOEt 50% fraction, using a SiOH column, yielded 4.7 mg of an yellow powder (Rf = 0.4 in silica gel eluted with C_6H_6:AcOEt:HCOOH (9:7:3)) (Compound **CT-1**) which was submitted to ^{1}H, ^{13}C NMR for structural identification. UV absorption data and MS (Negative ion mode) was later determined when injecting a sample in an HPLC coupled with diode array detector (DAD) and tandem mass spectrometer (MS/MS) and are herein discussed for structural determination.

This compound has shown a major absorption band in UV at 289 nm compatible with a flavanone structure (Markham and Mabry, 1975). The first order mass spectrum showed a pseudomolecular ion *m/z* 287 $[M-H]^-$ consistent with a tetrahydroxy-substituted flavanone, although position of hydroxyl groups was yet impossible to determine. NMR experiments were therefore needed for complete structural identification.

Thus, the ^{1}H-NMR spectrum revealed characteristic resonances of five aromatic protons such as δ7.32 ppm (d, *J*=8.8 Hz), c), δ 7.01 ppm (d, *J*=2.5 Hz), δ 6.81 ppm (d, *J*=8.8 Hz), δ 6.55 ppm (d, *J*= 8.8 Hz) and δ 6.88 ppm (dd, *J*= 8.8; 2.5 Hz).

From the 2D ^{1}H-^{1}H COSY experiment (Figure 3.1) and through the coupling constants (*J*) observed in Table 3.2, a three-protons spin system with only one *ortho* coupling constant (δ 7.01, d, *J*=2.5 Hz; δ 6.81, d, *J*=8.8 Hz; δ 6.88, dd, *J*= 8.8; 2.5 Hz) strongly suggested the presence of a 3',4'-disubstitution on the B ring. Additionally, the other two-aromatic protons spin system (δ7.32, d, *J*=8.8 Hz; δ 6.55, d, *J*=8.8 Hz) suggested an *ortho*-dihydroxy substitution pattern in A-Ring.

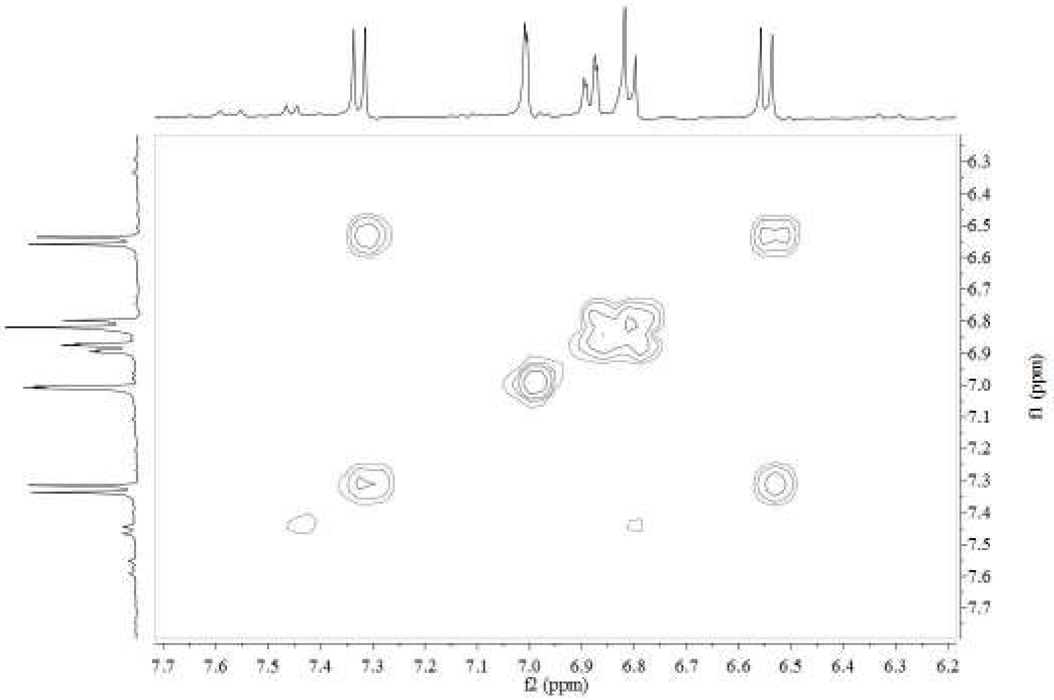


Figure 3.1. Detail of ^{1}H-^{1}H correlations displayed in the COSY spectrum of compound **CT-1**.

Furthermore, the presence in the ^{1}H-NMR spectrum of signals at δ 5.40 ppm (1H, dd, *J*=12.4, 3.1 Hz, $H_{2\beta}$), δ 3.10 (1H, dd, *J*=16.7, 12.4 Hz, $H_{3\alpha}$) and δ 2.75 (1H, dd, *J*=16.7, 3.1 Hz, $H_{3\beta}$), in conjunction with the ^{13}C-NMR signals at δ 80.2 and 43.7 ppm, along with the signal from the carbonyl group δ 192.6 ppm (Table 3.2), pointed to the presence of an -O-CH-CH_2-CO-system in the C-ring compatible with a flavanone structure (Hammami et al., 2004).

Analysis of HMQC and HMBC spectra and comparison of obtained data (Figure 3.2 and Table 3.2) with that reported in the literature (Foo, 1989; Clark-Lewis and Nair, 1964) allowed the identification of compound **CT-1** as 7,8,3’,4’-tetrahydroxyflavanone, also known as flavanokanin (**88**).

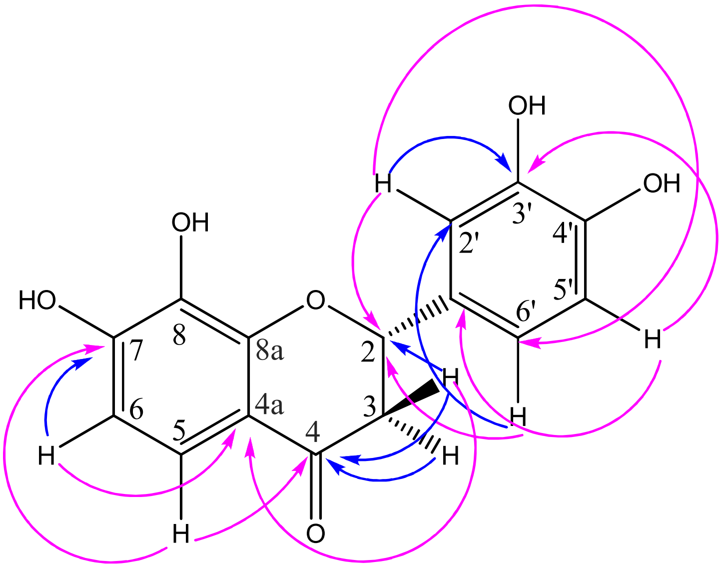


Figure 3.2. ${}^{2}J_{C\text{-}H}$ and ${}^{3}J_{C\text{-}H}$ correlations displayed in the HMBC spectrum of compound **CT-1**.

Table 3.2. Compound **CT-1,** ^{1}H and ^{13}C NMR data (400 MHz, δ ppm, (*J*) Hz, CD$_3$OD)

#	δ^{1}H, multiplicity, (*J*)	δ^{13}C	HMBC
2	5.40 dd (12.4; 3.1)	80.2	δ$_H$ 3.1; 6.88; 7.01
3	α: 3.10 dd (12.4; 16.7) β: 2.75 dd (3.1; 16.7)	43.7	
C=O		192.6	δ$_H$ 3.1; 2.75; 7.32
4a		132.6	δ$_H$ 6.55; 3.1
5	7.32 d (8.8)	117.9	
6	6.55 d (8.8)	109.5	
7		151.3	δ$_H$ 7.32; 6.55
8			
1		130.5	δ$_H$ 6.81
2	7.01 d (2.5)	113.6	δ$_H$ 6.88
3’		145.1	δ$_H$ 6.81; 7.01
4’			
5’	6.81 d (8.8)	114.8	
6’	6.88 dd (8.8; 2.5)	118.2	δ$_H$ 7.01

3.2.2. Structural identification of compound CT-2: Okanin (79)

Compound **CT-2** (15.7 mg), was also obtained from Hex:AcOEt 50 % fraction as an orange powder (Rf=0.75 in silica gel eluted with C_6H_6:AcOEt:HCOOH (9:7:3)). The molecular formula $C_{15}H_{12}O_6$ was established on the basis of MS analysis (*m/z* 287$[M-H]^-$) and ^{13}C NMR spectra. The UV absorption maximum at 380 nm indicated a chalcone skeleton.

The ^1H NMR spectrum of compound **CT-2** showed the presence of five aromatic protons: δ6.51 (d, *J*=9 Hz), δ6.86 (d, *J*= 8.2 Hz), δ7.15 (dd, *J*=8.2; 1.9 Hz), δ7.22 (d, *J*=1.9 Hz) and δ7.57 (d, *J*=9 Hz).

Additionally structural confirmation of chalcone skeleton was achieved by observing the ^1H NMR spectrum, which showed the characteristic chemical shifts and coupling constant of a chalcone's olefinic protons H_α (δ7.58, d, *J*=15 Hz) and of H_β (δ7.77, d, *J*=15 Hz) (Figure 3.3).

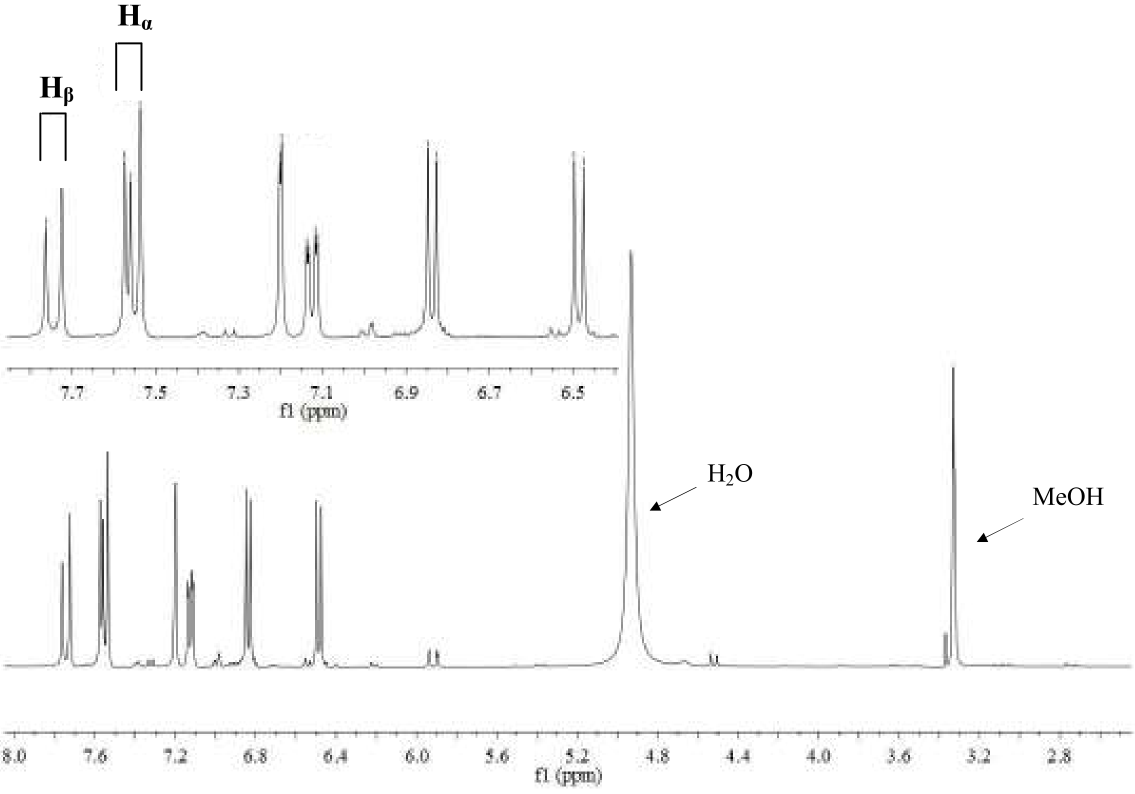


Figure 3.3. ^1H NMR spectrum (400 MHz; CD_3OD), with additional amplified aromatic region, of compound **CT-2**.

Proton homocorrelation (^1H-^1H COSY) experiment revealed two independent spin systems: the first one, just like the previous compound CT1, presented a three-protons spin system with merely one *ortho* coupling constant (δ 7.22, d, *J*=1.9 Hz; δ 6.86, d, *J*=8.2 Hz; δ 7.15, dd, *J*= 8.2; 1.9 Hz) which strongly indicated a 3,4-disubstituted B ring, and a second spin system (δ6.51, d, *J*=9.0 Hz; δ 7.57, d, *J*=9.0 Hz) which along with MS data (suggesting a total of six oxygen atoms) has led to believe that **CT-2** was likely to have an *ortho*-trihydroxy substitution pattern in A-Ring.

The ^{13}C NMR spectrum of compound CT-2 showing a carbonyl carbon at δ 194.1 ppm and signals at 118.4 and 146.1 typical of chalcone α and β-carbons, along with HMQC experiment data confirmed a chalcone structure.

The sequential assignments of protons and carbons atoms that would confirm the substitution pattern were mostly made with the help of long range couplings observed in the HMBC spectrum (Figure 3.4).

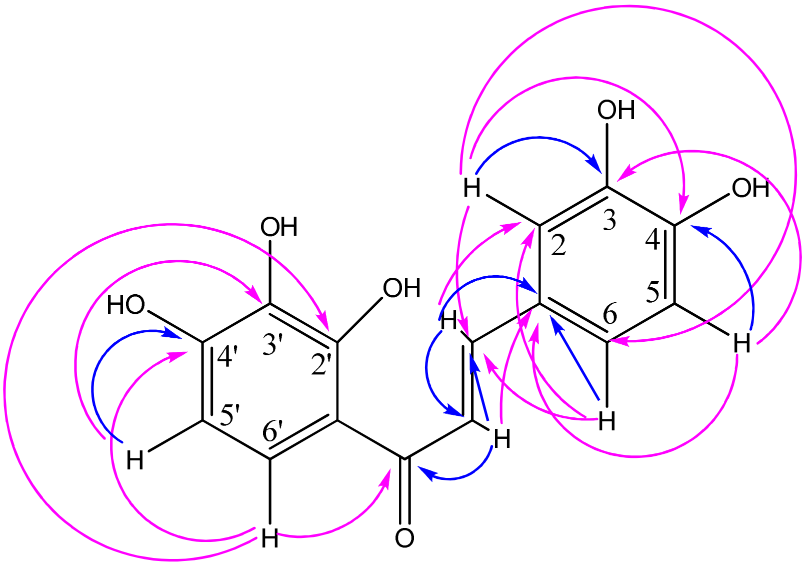


Figure 3.4. $^2J_{C\text{-}H}$ and $^3J_{C\text{-}H}$ correlations displayed in the HMBC spectrum of compound **CT-2**.

Based on the physical data from UV, MS, ^{13}C and ^1H NMR spectroscopy (Table 3.3) this compound was identified as okanin (**79**), already described for *C. tinctoria*

(Shimokoriyama and Hattori, 1953) and with spectroscopic data in accordance with the literature (Kuo et al., 2007).

Table 3.3. Compound **CT-2,** ^{1}H and ^{13}C NMR data (400 MHz, δ ppm, (*J*) Hz, CD_3OD)

#	δ^1H, multiplicity, (*J*)	δ^{13}C	HMBC
1		128.5	δ_H 7.77; 7.58; 6.86
2	7.22 d (1.9)	115.2	δ_H 7.77; 7.15
3		146.9	δ_H 7.22; 6.86
4		150.0	δ_H 7.22; 7.15; 6.86
5	6.86 d (8.2)	115.8	
6	7.15 dd (8.2; 1.9)	123.7	δ_H 7.77; 7.22
α	7.58 d (15.0)	118.4	δ_H 7.77
β	7.77 d (15.0)	146.1	δ_H 7.58; 7.22; 7.15
C=O		194.1	δ_H 7.77; 7.58; 7.57
1’		116.7	
2’		154.5	δ_H 7.57
3’		133.8	δ_H 6.51
4’		153.3	δ_H 7.57; 6.51
5’	6.51 d (9.0)	108.6	
6’	7.57 d (9.0)	123.2	

3.2.3. Structural identification of compound CT-3: Marein (78)

Compound **CT-3** was isolated from AcOEt 100% fraction (80 mg), which was fractionated by size exclusion sephadex column. The result was the isolation of a yellow powder (10 mg) from one of the subsequent fractions (Chapter 8). TLC control showed a single intense purple spot (Rf = 0.1 in silica gel eluted with C_6H_6:AcOEt:HCOOH (9:7:3)) after spraying with NP/PEG reagent. Therefore through MS data (*m/z* 449 [M-H]$^-$ and fragment *m/z* 287 [M-H-glucose]$^-$) along with UV (absorption maximum at 380 nm) a preliminary characterization was achieved: compound **CT-3** was very likely a glucosilated chalcone.

NMR experiments were subsequently preformed and the proton NMR showed signals for 14 protons of which 7 were aromatic or olefinic (δ^1H at 6-8 ppm) and 7 were aliphatic (δ^1H at 3-5 ppm). The two doublets (δ7.72 and δ7.68 ppm) with the *J*=15 Hz characteristic of *trans* configuration of the α and β protons confirmed compound **CT-3** as a chalcone (Redl et al, 1993). Also the number of aromatic protons and coupling pattern: three *ortho*-coupled protons: H5' (δ6.77, d, *J*=9.2 Hz), H6'(δ7.78, d, *J*=9.2 Hz) and H5 (δ6.83, d, *J*=8.2 Hz); one *meta*-coupled proton: H2 (δ7.30, d, *J*=2.0 Hz) and one *ortho*, *meta*-coupled proton H6 (δ7.23, dd, *J*= 8.2; 2.0 Hz), like that observed for CT-2, were compatible with a 2',3,3',4,4'-pentahydroxychalcone structure.

The other 7 aliphatic proton-signals along with the previously analysed MS data indicated that they probably belonged to a coupled sugar molecule. The signal at 4.92 ppm showed the characteristic chemical shift and coupling constant (*J*=7.2 Hz) of the anomeric proton H-1'' of a beta-glucose (Redl et al, 1993).

The assignment of the proton-signals to the structure was accomplished by comparison of the recorded data with the literature values (Redl et al., 1993; Hoffmann and Holzl, 1989; Hoffmann and Holzl, 1988) and with the help of 2D ^{1}H-^{1}H COSY experiment. The homocorrelation spectrum (2D ^{1}H-^{1}H COSY) was compatible with 2',3',4' substitution pattern of the A-Ring and 3,4 substitution pattern of the B-ring of the chalcone.

Final assignment of the protons to the carbons was established with the help of 2D HMQC which along with the literature values (Redl et al., 1993; Hoffmann and Holzl, 1988) made it possible to identify the glucose as the sugar-molecule linked to the chalcone.

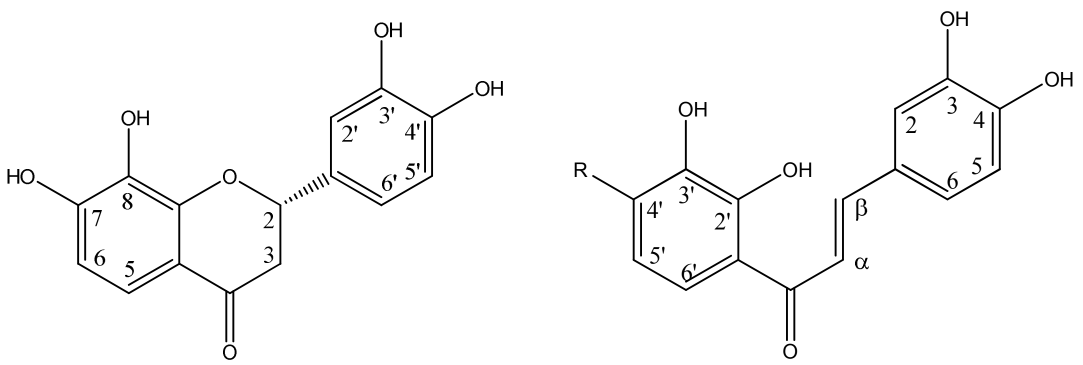
Finally, although it was not possible to obtain the total ^{13}C NMR spectrum, the 2D HMBC spectrum allowed the complete assignment of chalcone-skeleton carbons (Table 3.4). Altogether, compound **CT-3** was identified as an okanin-O-glucoside.

HMBC experiment showed no long-distance correlations between the H-1'' of the glucose and a carbon of the chalcone-skeleton so, the position of the beta-glucose could not be assigned through recorded spectral data. Considering that marein (okanin-4'-O-glucoside was already identified in *C. tinctoria* (Shimokoryiama, 1957; Zhang et al., 2006), the chemical shifts of compound **CT-3** were compared with those in the literature (Hoffman and Holzl, 1989) and together with HPLC-DAD-MS/MS data (Section 3.2.2), compound **CT-3** was then identified as marein (**78**).

Table 3.4. Compound **CT-3,** ^{1}H and ^{13}C NMR data (400 MHz, δ ppm, (*J*) Hz, DMSO-d6)

#	δ^{1}H, multiplicity, (*J*)	δ^{13}C	HMBC
1		126.0	δ$_H$ 6.83; 7.70
2	7.30 d (2.0)	116.4	δ$_H$ 7.23
3		146.0	δ$_H$ 6.83
4		149.5	δ$_H$ 7.23; 7.30
5	6.83 d (8.2)	116.1	
6	7.23 dd (8.2; 2.0)	122.9	δ$_H$ 7.30
α	7.70 d (15.4)	117.8	
β	7.72 d (15.4)	145.8	δ$_H$ 7.23; 7.30
C=O		192.5	δ$_H$ 7.70; 7.72; 7.78
1’		118.0	δ$_H$ 7.70; 6.77
2’		153.0	δ$_H$ 7.78
3’		135.0	δ$_H$ 6.77
4’		151.0	δ$_H$ 7.78
5’	6.77 d (9.2)	106.8	
6’	7.78 d (9.2)	121.9	
1’’	4.92 d (7.2)	101.3	
2’’	3.72	74.0	
3’’	3.38	76.0	
4’’	3.18	71.0	
5’’	3.45	78.0	
6’’a	3.72 d (11.6)	61.0	
6’’b	3.57		

In summary, the three compounds isolated are represented in Figure 3.5. Stereochemical structure of flavanokanin was attributed based on the biosynthesis.



(**88**)

(**79**) R= OH
(**78**) R= O-Glucose

Figure 3.5. Chemical structure of the three compounds isolated; flavanokanin (**88**), okanin (**79**) and marein (**78**).

Parallel to this phytochemical study, *in vivo* experiments were performed yielding results that supported the claimed antihyperglycemic activity. Therefore, in order to determine which compounds were responsible for such pharmacological activity, a flavonoid-rich fraction was prepared from direct liquid-liquid fractionation of the aqueous extract with ethyl acetate (plant material ***CTD1***, AcOEt fraction sample A), as described in the experimental section (Chapter 8; Section 8.2.2). This fraction is different from the one prepared for compound isolation (AcOEt 100%, which was preceded by another liquid-liquid partitioning with hexane:AcOEt 50%). So from this section on, the AcOEt fraction mentioned refers to the more inclusive flavonoid-rich fraction.

3.3 Chemical characterization of extracts by HPLC-DAD-MS/MS

Qualitative analysis of *Coreopsis tinctoria* aqueous extract and AcOEt fraction was performed in order to identify the active principles responsible for pharmacological activity claimed for this plant.

A number of preliminary HPLC experiments, employing different elution systems and samples of *C. tinctoria* flowering tops aqueous extract and AcOEt fraction, were preformed in order to establish optimal conditions for HPLC analysis of this species. Once chosen the adequate HPLC conditions (Chapter 8), a chemical profile for fresh infusions (Sample C) and one-week old infusion (Sample C) and AcOEt fraction (Sample B) was obtained, as shown in the chromatograms presented in Figure 3.6 and 3.7 respectively.

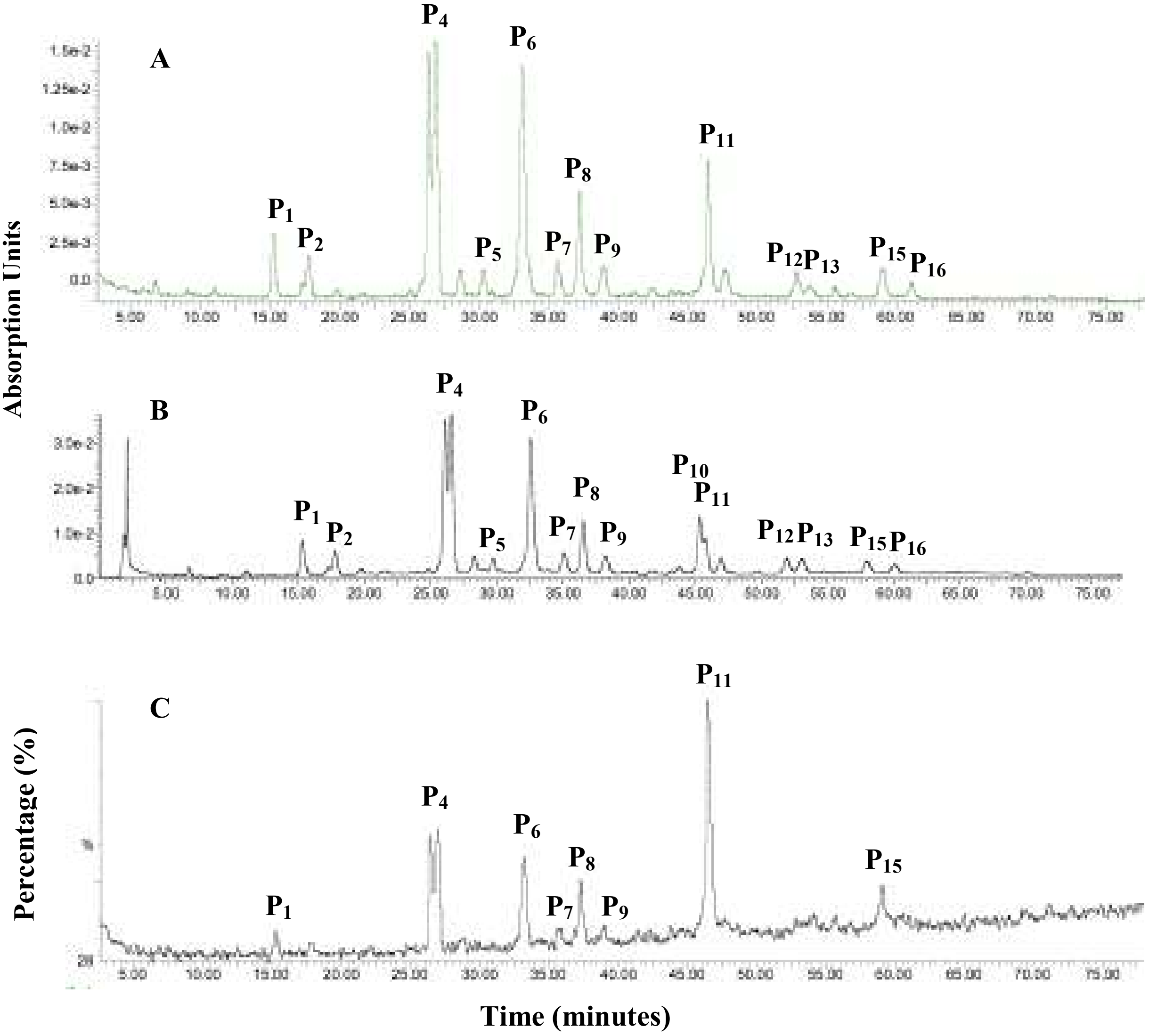


Figure 3.6. HPLC chromatographic profile of *C. tinctoria* flowering tops aqueous extract. **A.** UV chromatogram at 280 nm of fresh aqueous extract (Sample C). **B.** UV chromatogram at 280 nm of one-week old aqueous extract (Sample C). **C.** Total ion chromatogram of fresh aqueous extract (Sample C).

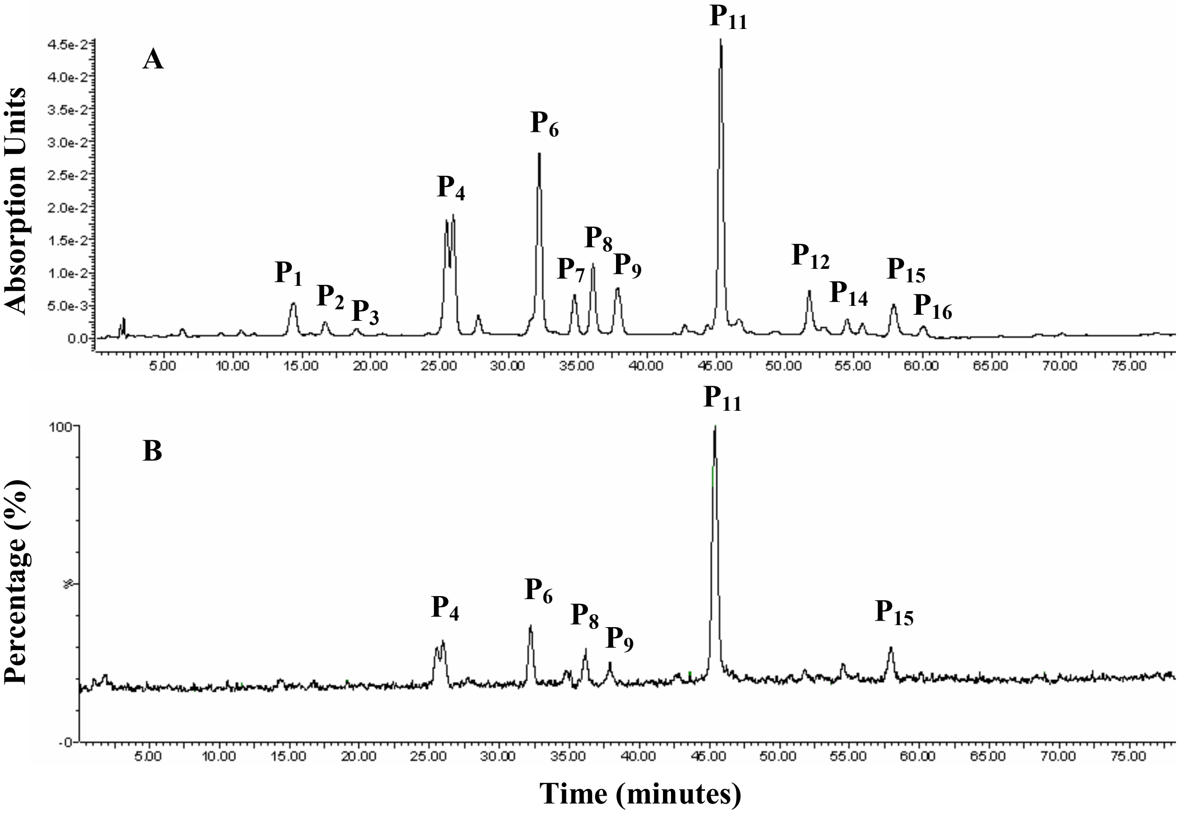


Figure 3.7. HPLC chromatographic profile of *C. tinctoria* flowering tops AcOEt fraction (Sample B). **A** – UV chromatogram at 280 nm; **B** – Total ion chromatogram.

From the chromatograms it is possible to observe good peak resolution which, with the help of the coupled diode array detector (DAD) and tandem mass spectrometer (MS/MS), a total of 16 compounds were identified (Table 3.5 and Figure 3.8).

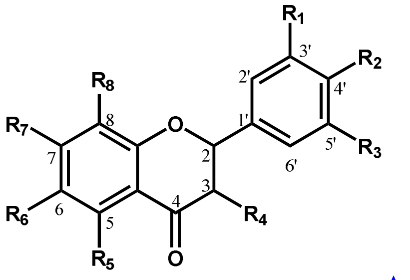
Compounds detected were mainly aglycones and glycosides of flavanone and chalcone flavonoid types (structures A and C in Figure 3.8). Peak **P_{11}** appears as the major compound detected in the total ion chromatogram of both aqueous extracts and AcOEt fractions (Figure 3.6 C and 3.7 B).

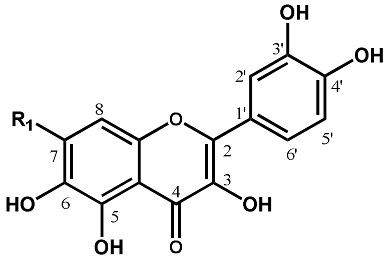
Among the 16 compounds characterized, six of these compounds (peaks **P_2**, **P_4**, **P_6**, **P_8**, **P_{11}** and **P_{15}**) were unambiguously identified by comparing retention times (Rt), UV and MS data with those of the reference standards whereas the other 10 peaks detected in the chromatogram of *C. tinctoria* extracts were tentatively identified on the basis of data from literature and analyzing their fragmentation patterns. Peaks **P_1, P_3, P_9** and **P_{12}** are reported in *C. tinctoria* for the first time.

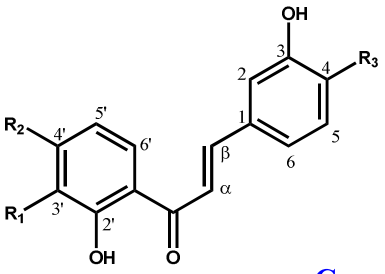
Table 3.5. Peak assignment, retention time (Rt), UV and mass spectral data of compounds detected in *Coreopsis tinctoria* flowering tops aqueous extract and AcOEt fraction.

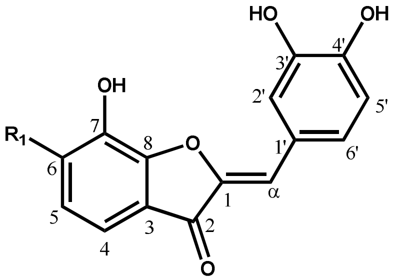
Peak	Rt (min)	Compound Identification	Uv max (nm)	[M-H]$^-$	Daughter ions
P$_1$	14.35	3,4’,5,6,7-pentahydroxyflavanone-O-glucoside (**93**)	285	465	303 [M-H-Hexose]$^-$, 285 [M-H-Hexose-H$_2$O]$^-$
P$_2$	16.63	Chlorogenic Acid (**28**)	290sh, 323	353	191 [M-H-Caffeic Acid]$^-$
P$_3$	19.80	3,3’,5,5’,7-pentahydroxyflavanone-O-glucoside (**94**)	285	465	303 [M-H-Hexose]$^-$
P$_4$	25.91	Flavanomarein (**85**)	283	449	431 [M-H-H$_2$O]$^-$, 287 [M-H-Glucose]$^-$, 269 [M-H-Glucose-H$_2$O]$^-$, 151 [^{1,3}A$^-$], 135 [^{1,3}A$^-$ -16]$^-$
P$_5$	29.00	Flavanocorepsin (**95**)	285	433	271 [M-H-Glucose]$^-$
P$_6$	32.14	Flavanokanin (**88**)	289	287	151 [^{1,3}A$^-$], 135 [^{1,3}A$^-$ -16]$^-$
P$_7$	35.00	3,4’,5,6,7-pentahydroxyflavanone(**90**)	288	303	285 [M-H-H$_2$O]$^-$
P$_8$	36.02	Quercetagetin-7-O-glucoside (**86**)	357	479	317 [M-H-Glucose]$^-$
P$_9$	37.83	3’,5,5’,7-tetrahydroxyflavanone-O-glucoside (**96**)	285	449	287 [M-H-Hexose]$^-$
P$_{10}$	45.00	Maritimein (**80**)	419	447	285 [M-H-Hexose]$^-$
P$_{11}$	45.29	Marein (**78**)	380	449	431 [M-H-H$_2$O]$^-$, 287 [M-H-Glucose]$^-$, 269 [M-H-Glucose-H$_2$O]$^-$, 151 [^{1,3}A$^-$], 135 [^{1,3}A$^-$ -16]$^-$
P$_{12}$	51.70	Dicaffeoylquinic acid (**97**)	300sh, 328	515	353 [M-H-Caffeic Acid]$^-$
P$_{13}$	53.16	Okanin aurone (**98**)	414	285	
P$_{14}$	54.44	Coreopsin (**76**)	381	433	271 [M-H-Glucose]$^-$, 135 [^{1,3}A$^-$]
P$_{15}$	57.82	Okanin (**79**)	380	287	151 [^{1,3}A$^-$], 135 [^{1,3}A$^-$ -16]$^-$
P$_{16}$	59.97	3’,5,5’,7-tetrahydroxy flavanone (**91**)	285	287	151 [^{1,3}A$^-$]

Peak	Compound Structure	R1	R2	R3	R4	R5	R6	R7	R8	Compound
P_1	A	H	OH/OG	H	OH/OG	OH/OG	OH/OG	OH/OG	H	**93**
P_2	E	OH	OH	OH	-	-	-	-	-	**28**
P_3	A	OH/OG	H	OH/OG	OH/OG	OH/OG	H	OH/OG	H	**94**
P_4	A	OH	OH	H	H	H	H	OG	OH	**85**
P_5	A	OH	OH	H	H	H	H	OG	H	**95**
P_6	A	OH	OH	H	H	H	H	OH	OH	**88**
P_7	A	H	OH	H	OH	OH	OH	OH	H	**90**
P_8	B	OG	-	-	-	-	-	-	-	**86**
P_9	A	OH/OG	H	OH/OG	H	OH/OG	H	OH/OG	H	**96**
P_{10}	D	O/OG	-	-	-	-	-	-	-	**80**
P_{11}	C	OH	OG	OH	-	-	-	-	-	**78**
P_{12}	E	Caffeic acid or OH	Caffeic acid or OH	Caffeic acid or OH	-	-	-	-	-	**97**
P_{13}	D	OH	-	-	-	-	-	-	-	**98**
P_{14}	C	H	OG	OH						**76**
P_{15}	C	OH	OH	OH	-	-	-	-	-	**79**
P_{16}	A	OH	H	OH	H	OH	H	OH	H	**91**


A


B


C


D

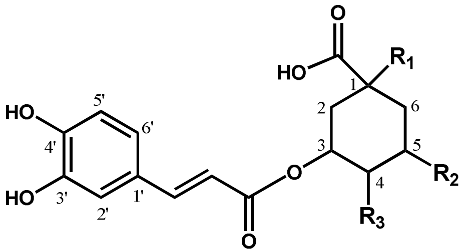

E

Figure 3.8. Summary of the chemical structures of compounds identified in *C. tinctoria's* flowering tops aqueous extract and AcOEt fraction. **A** – Flavanone; **B** – Flavonol; **C** – Chalcone; **D** – Aurone; **E** - Phenyl-propanoic acid. OG = O-Glucose.

When analysing the chromatograms presented in Figure 3.6, representing the chemical profile of the fresh aqueous extract (Figure 3.6 A) and the one-week old aqueous extract (Figure 3.6 B) it is possible to observe some differences. The most obvious one can be seen arround $\mathbf{P_{11}}$, marein (identified by comparison with standard: Rt = 45.29 min.; UV max. = 380 nm; *m/z* 449), which in the fresh extract is presented as a single peak (Rt = 45.29 min.) while in the old extract an adjacent, nearly overlapping, peak arises: $\mathbf{P_{10}}$ (Rt = 45.00 min.). $\mathbf{P_{10}}$ UV absorption maximum at 419 nm was indicative of an aurone skeleton (Figure 3.9). In addition, the molecular ion *m/z* 447 $[M\text{-}H]^-$ lacked 2 protons when comparing to adjacent marein ($\mathbf{P_{11}}$) which could be derived from its oxidation process (Figure 3.11), generally accepted for aurone biosynthesis (Veitch and Grayer, 2006). Thus, $\mathbf{P_{10}}$ was tentatively identified as maritimein (**80**), previously detected in this species (Shimokoriyama, 1957). The presence of this chalcone-aurone pair is consistent with the pattern described for the genus *Coreopsis* (Harborne and Gueissman, 1956).

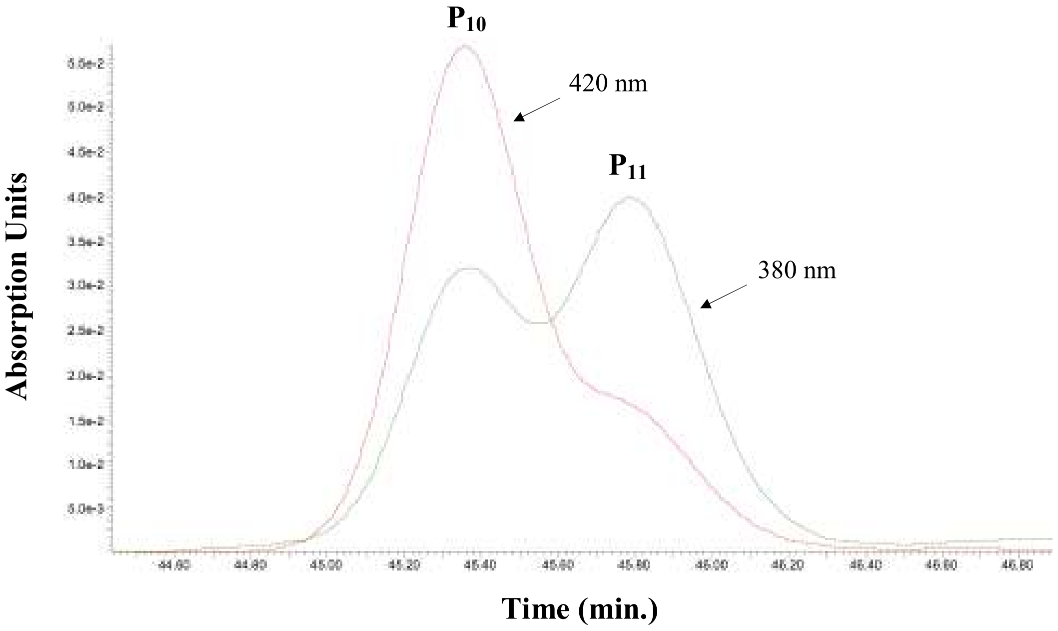


Figure 3.9. Detail of peaks $\mathbf{P_{10}}$ and $\mathbf{P_{11}}$ present in the one-week old aqueous extract chromatogram at two different wavelengths 420 nm and 380 nm ($\mathbf{P_{10}}$ and $\mathbf{P_{11}}$ absorption maximums respectively).

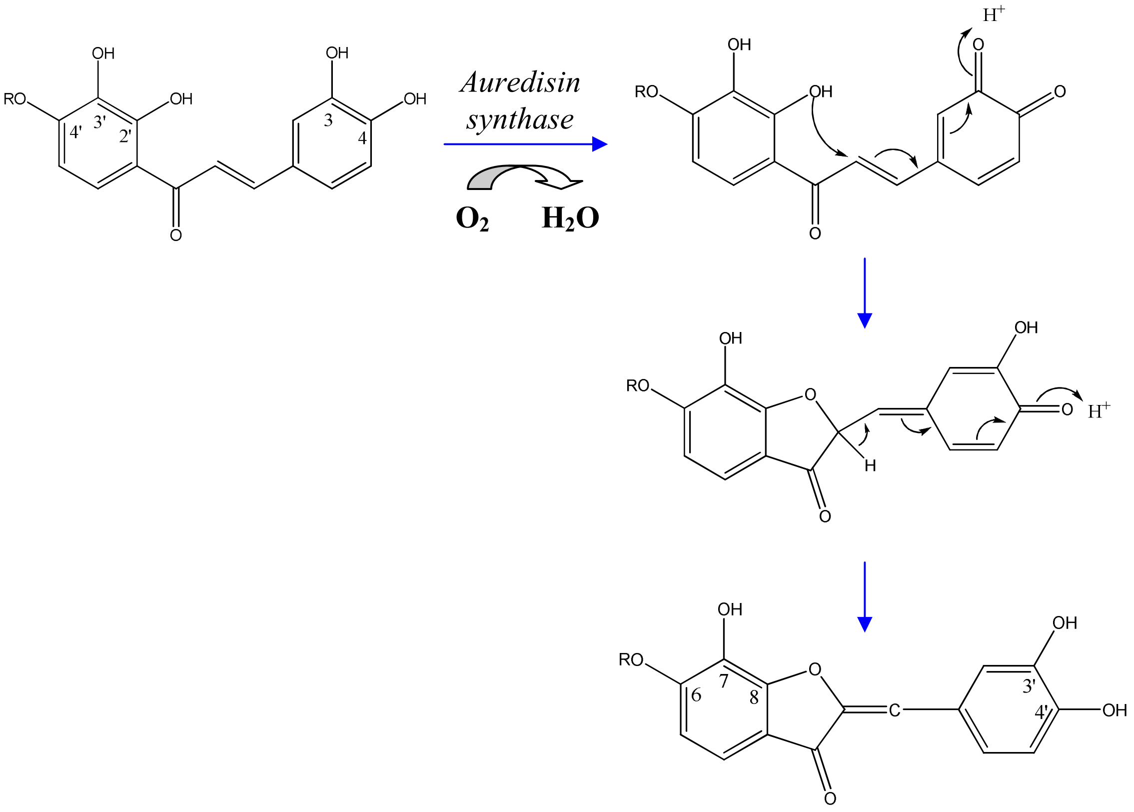
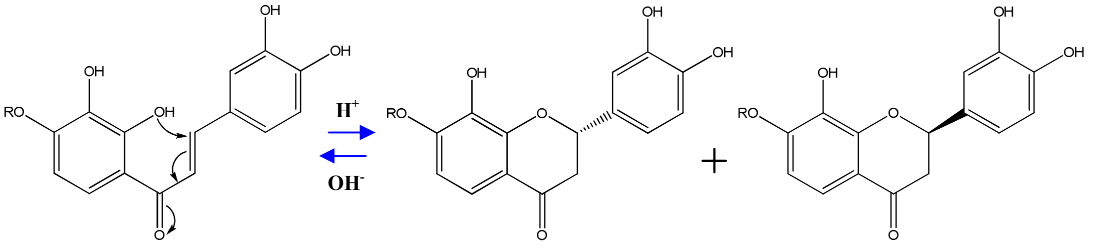


Figure 3.10. Mechanism of chemical or enzymatic chalcone oxidation to the correspondent aurone according to Veitch and Grayer (2006). R=Glucose.

As for $\mathbf{P_{10}}$, it was not detected in the AcOEt fraction, which might be related to preservation of the samples. This fraction was prepared from direct liquid-liquid fractionation, the solvent (AcOEt) was evaporated and the resulting solid was stored in a desiccator. On the contrary, the one-week old aqueous extract, where $\mathbf{P_{10}}$ is visible, was stored in H_2O/MeOH solution for a week in the fridge, which could promote $\mathbf{P_{11}}$ oxidation and correspondent $\mathbf{P_{10}}$ formation (Figure 3.10).

Similar behaviour concerning data from MS (with a loss of 2 protons in the pseudomolecular ion) and UV (with an absorption maximum at 380 nm for one compound and 414 nm for the other) and was observed between compound peaks $\mathbf{P_{15}}$ and $\mathbf{P_{13}}$ respectively. Therefore another chalcone-aurone pair was suggested. $\mathbf{P_{15}}$ was successfully identified comparing data from the previously isolated compound **CT-2** okanin (**79**), already identified in this species (Shimokoriyama, 1957; Geissman et al., 1956) and compound peak $\mathbf{P_{13}}$ was then tentatively identified as okanin aurone (**98**).

Another abundant peak, observed in all samples, is peak compound **P$_4$** (Rt = 25.91 min.) which was identified as the natural isomer 2*S*-3',4',8-trihydroxyflavanone-7-O-glucoside (flavanomarein) by comparison of Rt with a commercial sample of the compound (purity ≥ 99%). The close and as abundant peak at Rt 25.5 min., presenting the same UV and MS spectra as **P$_4$**, was tentatively identified as the diasterioisomer 2*R*-3',4',8-trihydroxyflavanone-7-O-glucoside, a non-natural compound probably formed during extraction by chemical ring closure of the correspondent chalcone, marein (**78**) (Figure 3.11).



(78) **2S – (85)** **2R**

Figure 3.11. Chemical mechanism of formation of isomers 2*S* and 2*R*-flavanomarein from marein. R=Glucose.

As seen in figure 3.11, flavanomarein (**P$_4$**) is formed directly from a chalcone precursor, in this case marein (**P$_{11}$**). This inter-conversion is well known as biogenetically, chalcones are the immediate precursors of flavanones, and some flavanones isomerize by ring opening into chalcones during isolation from plants or after chemical treatment with alkali and vice-versa: chalcones isomerize into flavanones when treated with acids (Grayer and Veitch, 2006). As isomers, **P$_4$** and **P$_{11}$** share the same mass and so the proposed fragmentation pattern of the pair marein and flavanomarein is discussed together and is represented in Figure 3.12.

Both **P$_4$** and **P$_{11}$** were identified by comparison with commercial standards and according with the fragments obtained it could be confirmed that in the negative mode, flavanones and chalcones showed the same fragmentation routes (fragments detected in table 3.5) and no significant distinctions were observed. Also, an equilibrium state between chalcone and flavanone could be quickly obtained during their deprotonation process (Zhang et al., 2008).

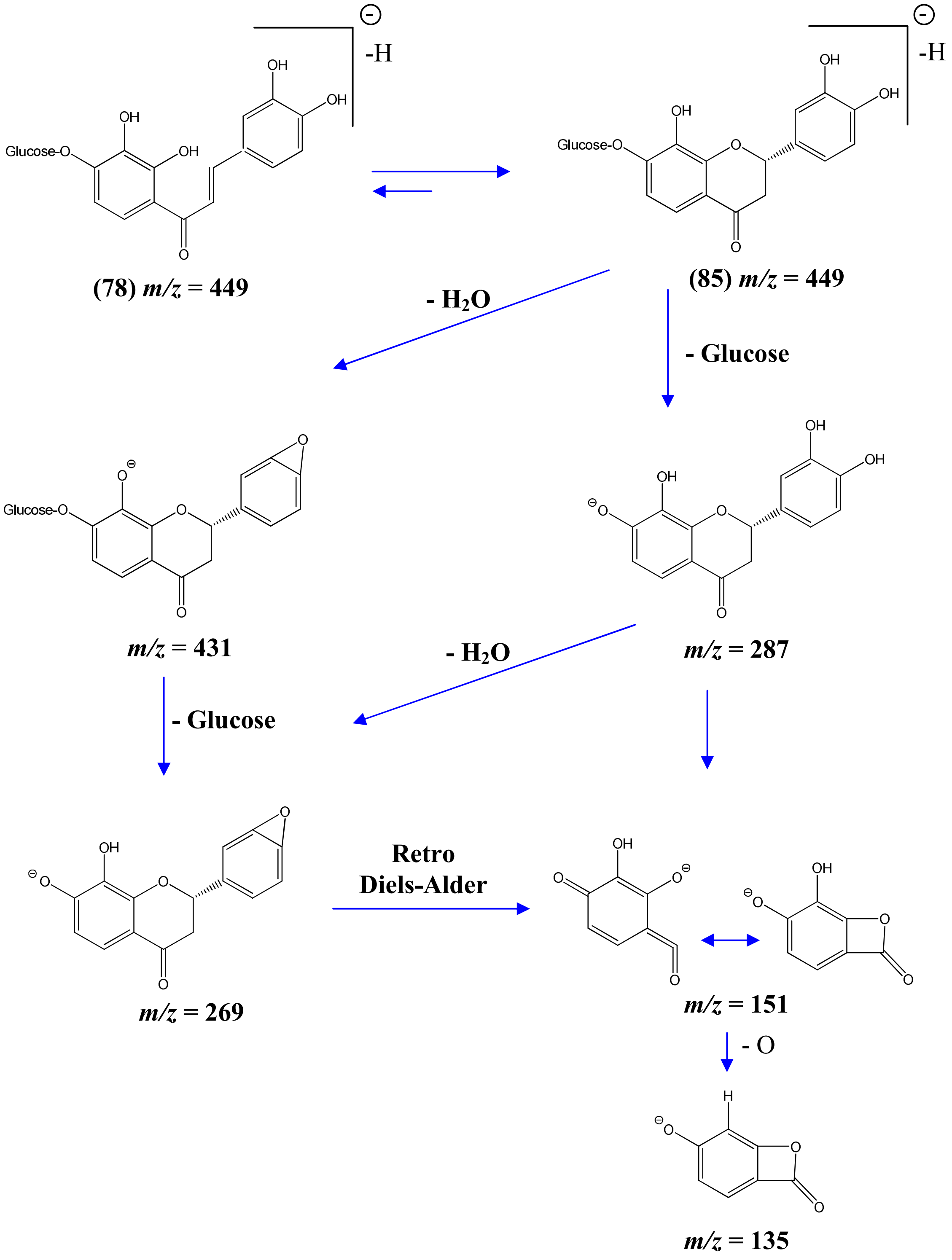


Figure 3.12. Proposed fragmentation for chalcone **78** (**P$_{11}$**) and flavanone **85** (**P$_4$**) pair in negative ion mode**.**

From the fragmentation of *m/z* 449 (daughter scan mode) the resulting main product ions were *m/z* 287 for both parent ions, corresponding to the loss of the glucose moiety (162 a.m.u.). However they also showed a small daughter ion at *m/z* 431 indicating a H_2O molecule loss (18 a.m.u.), characteristic of *ortho* dihydroxyl groups, and prior to the loss of glucose moiety. Another small fragment *m/z* 269 was observed and can be explained as the loss of H_2O after the loss of glucose moiety from parent ion *m/z* 287 (Figure 3.12).

Previous reports state that, when using negative ion mode, to analyze flavanones without hydroxyl substitution at $C_{2'}$, the main fragmentations are obtained from retro Diels Alder cleavages of C-ring, which produce a base peak assigned as $^{1,3}A^-$ but a rather low or even absent $^{1,3}B^-$ fragment (Zhang et al., 2008). This has been justified as a charge driven mechanism, where the resulting fragment ($^{1,3}B^-$) becomes neutral and is subsequently undetectable in this mode (Zhang et al., 2008). In this work, fragments *m/z* 151 and 135 were observed which could therefore correspond to ring A fragment ($^{1,3}A^-$) and a molecule resulting from the loss of an Oxygen atom (16 a.m.u.) of that same fragment (Figure 3.12). The possibility of fragment *m/z* 135 to originate from fragment *m/z* 151 was confirmed when this later fragment decreased in intensity while analysing the original marein sample using increasing collision energies, as seen in Figure 3.13 from D (lower energy) to A (higher energy) (Figure 3.13).

Peaks at retention time of 14.3 (**P_1**) and 19.8 min (**P_3**) showed a typical flavanone UV absorption maximum at 285 nm (Markham and Mabry, 1975). The same pseudomolecular ion *m/z* 465 ($[M-H]^-$) was observed for both compounds and these results are compatible with two pentahydroxyflavanone hexosides. From the fragmentation of *m/z* 465 (daughter scan mode) the resulting main product ions were *m/z* 303 for both parent ions, corresponding to the loss of the hexose moiety (162 a.m.u.). However compound **P_1** also showed a daughter ion at *m/z* 285, indicating a H_2O molecule loss (18 a.m.u.), which was already seen as a characteristic fragment of molecules with *ortho* dihydroxyl groups (Wang et al., 2008). Peak compounds **P_1** and **P_3** were tentatively identified as 3,4',5,6,7-pentahydroxyflavanone glucoside (**93**) and 3,3',5,5',7-pentahydroxyflavanone glucoside (**94**), respectively.

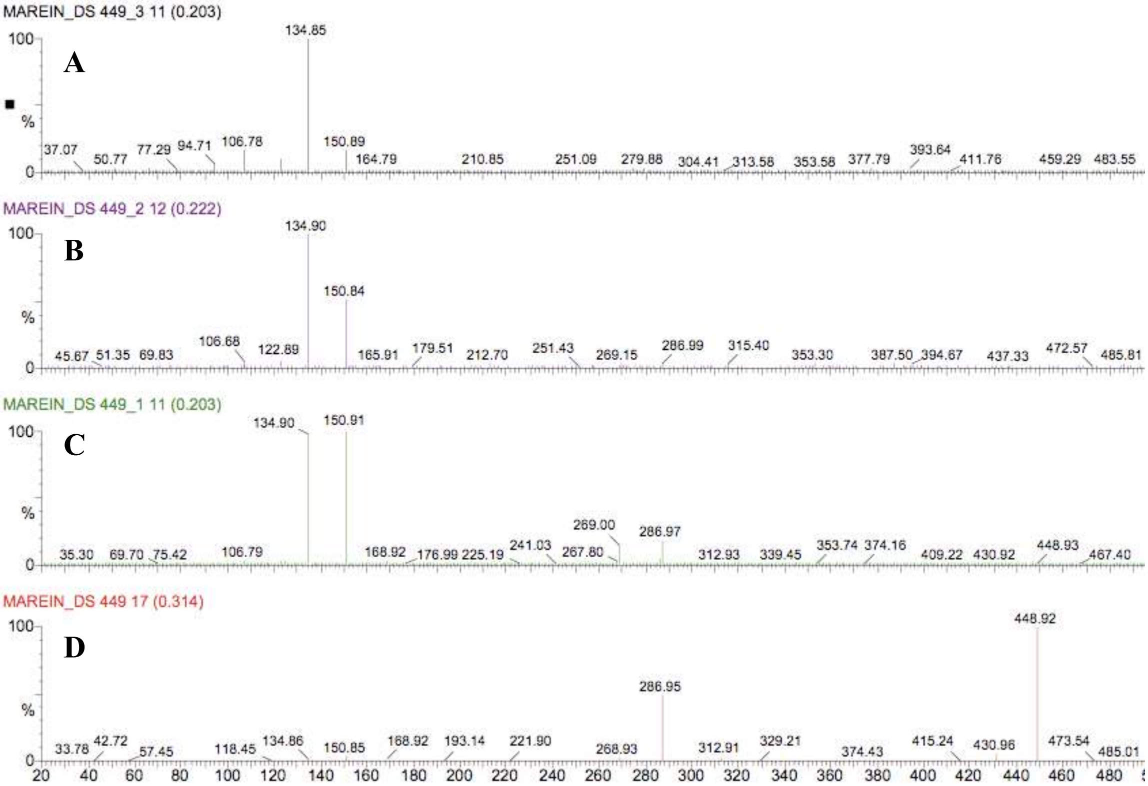


Figure 3.13. Fragments obtained from marein (**78**) in negative ion mode using different collision energies **A**- highest to **D**- lowest.

All previously identified glycosilated-flavonoids present in *C. tinctoria* had, as sugar moiety, glucose molecules. Additionally, glucose being the most abundant hexose in nature led to the assumption that the tentatively identified hexosides, probably were glucosides. The corresponding aglycones (**90** and **89**) were already described for the flowers of *C. tinctoria* (Zhang et al., 2006) but not in the corresponding glycosidic forms. Aglycone of compound corresponding to peak $\mathbf{P_1}$ was also tentatively identified in our sample (compound $\mathbf{P_7}$; Rt 35 min.).

The UV spectra of peak compounds $\mathbf{P_9}$ and $\mathbf{P_{16}}$ indicated a flavanone structure and the MS a tetrahydroxyflavanone hexoside and aglycone, respectively, as in the case of flavanomarein ($\mathbf{P_4}$) and flavanokanin ($\mathbf{P_6}$). The MS/MS of peak $\mathbf{P_{16}}$ presented only one daughter ion at *m/z* 151, assigned to $^{1,3}A^-$ (Zhang et al., 2008), which implicated the presence of two OH groups in the A ring. Since the compound 2*S*-3’,5,5’,7-

tetrahydroxyflavanone was already identified in this species (Zhang et al., 2006), peaks $\mathbf{P_9}$ and $\mathbf{P_{16}}$ were tentatively identified as the glucosidic form, 3',5,5',7-tetrahydroxyflavanone-O-glucoside (**96**), and corresponding aglycone (**91**), respectively.

UV absorption maxima of compound peaks $\mathbf{P_2}$ (Rt 16.63 min.) and $\mathbf{P_{12}}$ (Rt 51.7 min.) were around 320 nm with a shoulder near 290-300 nm, compatible with phenylpropanoic acid structures. The first order MS spectra of $\mathbf{P_{12}}$ showed the molecular ion *m/z* 515 ($[M-H]^-$), whereas MS/MS spectrum was very similar to spectrum of chlorogenic acid standard ($\mathbf{P_2}$): a fragment ion at *m/z* 353 corresponding to the loss of a caffeic acid molecule and equivalent to $[M-H]^-$ of chlorogenic acid was detected and is described as a characteristic of a dicaffeoylquinic acid. Apart from chlorogenic acid ($\mathbf{P_2}$) no other caffeoylquinic acid had been appointed to *C. tinctoria* flowers yet.

Compound peak $\mathbf{P_{14}}$ (Rt 54.44 min.) shows typical chalcone UV maximum absorption band at 381 nm (Markham and Mabry, 1975). Mass spectral analysis of this compound revealed the presence of pseudomolecular ion *m/z* 433 compatible with a tetrahydroxychalcone glucoside. The MS/MS spectrum also revealed two fragments corresponding to the aglycone (*m/z* 271) and $^{1,3}A^-$ (*m/z* 135). Chalcone $\mathbf{P_{14}}$ was tentatively identified as coreopsin (**76**), already described in this species (Harborne, 1977). Like marein was easily transformed into its flavanone isomer, flavanomarein, the same was expected to happen with chalcone coreopsin. So when $\mathbf{P_5}$ (Rt = 29.00 min.) presented the same pseudomolecular ion *m/z* 433 ($[M-H]^-$), an UV maximum at 285 nm and the same fragmentation pattern *m/z* 271 (loss of a glucose moiety) $\mathbf{P_5}$ was tentatively identified as flavanocoreopsin (**95**). It is the first time this compound is described in *C. tinctoria*.

As for compound peak $\mathbf{P_8}$ (Rt = 36.02 min.), it presented a pseudomolecular ion of *m/z* 479 $[M-H]^-$ which along with its UV absorption maximum at 357 nm was compatible with a pentahydroxy-substituted glycosilated flavonol. Comparing the retention time and fragmentation pattern (*m/z* 317 $[M-H-Glucose]^-$) of $\mathbf{P_8}$ with commercial standard Quercetagetin-7-O-glucoside (**86**), a perfect match was observed.

3.4. HPLC-UV quantification of marein in *Coreopsis tinctoria* extracts

Quantification of bioactive principles through modern analytical tools is essential for establishing the authenticity, creditability, prescription and usage of traditional herbal formulations (Govindarajan et al., 2007a). A simple high-performance liquid chromatography (HPLC) method (**Method 1**) for the separation and quantitative determination of the apparent major compound of *C. tinctoria* fresh extracts, the chalcone marein (**78**), has been developed. Validation of the method was done in order to demonstrate its selectivity, linearity, precision, accuracy and limits of detection and quantification.

In order to characterise the extracts used in the biological assays, we developed and validated an HPLC-UV method for the quantification of marein, using its maximal absorbance wavelength, 380 nm, for best sensitivity. The HPLC-UV chromatogram of *C. tinctoria* infusion (sample A) revealed the presence of a main compound with the same retention time as a commercial sample of marein (**78**) (Figure 3.14). Also, co-chromatography of the extract with marein (**78**) revealed no additional chromatographic peaks and an increased absorbance of main peak at 6.44 min was observed. The method was shown to be linear in the range 10-100 µg/mL ($y = 556698x + 239957$ [x= concentration (µg/mL), y= peak area]; $r^2 = 0.9937$) (Figure 3.15), precise (RSD < 4%) (Table 3.6 and 3.7), accurate (Table 3.8) with an average recovery of 102 % and with appropriate limits of detection (LOD = 3.2 µg/mL) and quantification (LOQ = 9.8 µg/mL).

Marein (**78**) content in infusions, freshly prepared from the liophilized aqueous extract (samples A and B as described in Chapter 8, section 8.6) of *C. tinctoria* was determined to be approximately 4 % (w/w dry extract) (Table 3.9). Marein concentration was also determined in the AcOEt fraction (sample A) and was approximately 4 times more concentrated when comparing with the infusion. This information was taken into account when determining the doses to be used in the *in vivo* assays.

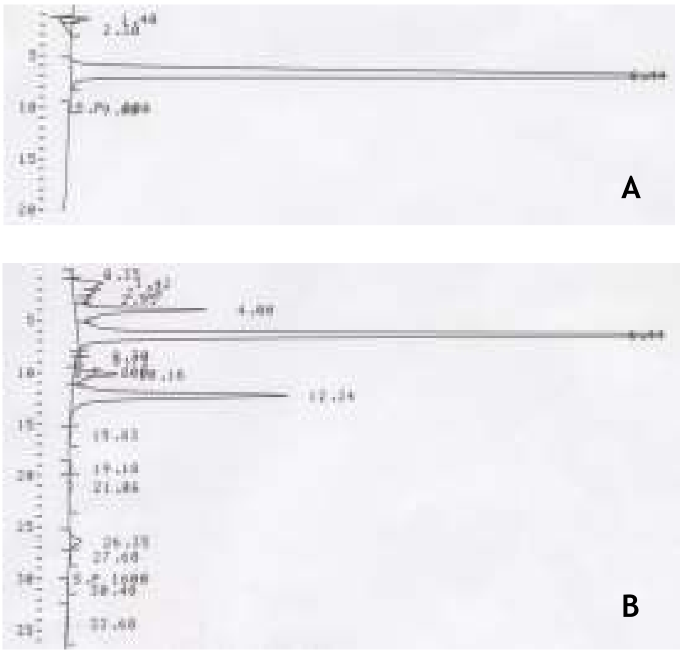


Figure 3.14. **A**-Chromatogram of the standard, marein (50 µg/mL), Rt 6.44 min., at 380 nm. **B**- Chromatogram of aqueous extract (Sample A) of *C. tinctoria* (1 mg/mL), Rt (main peak) 6.44 min, at 380 nm.

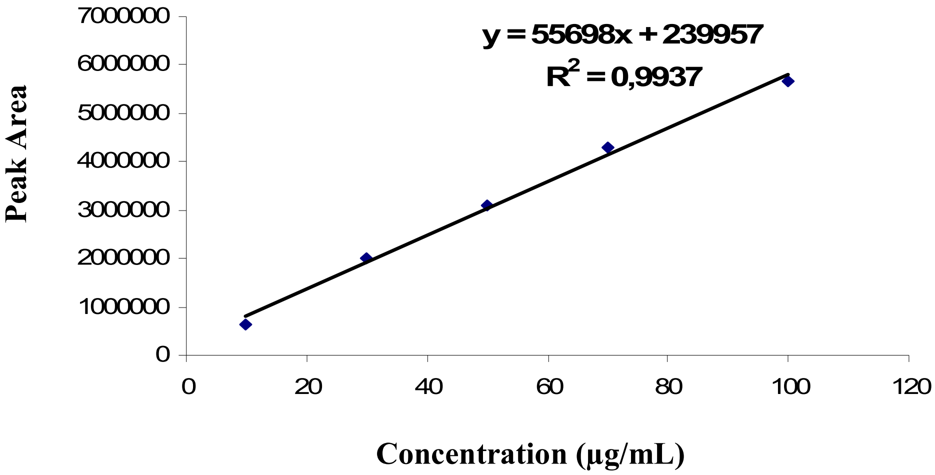


Figure 3.15. Correlation between peak area and marein concentration in the linearity range 10 µg/mL – 100 µg/mL.

Table 3.6. Precision determination: Method repeatability. SD: Standard deviation; RSD: Relative standard deviation.

Concentration (µg/mL)	**Average Peak Area**	**SD**	**RSD%**
10	628133	14683.79	**2.34**
30	2012099	69858.59	**3.47**
50	3087471	98143.68	**3.18**

Table 3.7. Precision determination: Inter-day precision. SD: Standard deviation; RSD: Relative standard deviation.

Concentration (µg/mL)	**Peak Área Day 1**	**Peak Área Day 2**	**Average**	**SD**	**% RSD**	**Average %RSD**
10	628133	568118	598125	30007.67	**5,02**	**< 4%**
30	2012099	1939844	1975971	36127.33	**1,83**	
50	3087471	2787211	2937341	150129.80	**5,11**	

Table 3.8. Accuracy determination.

Reference added (µg)	**Expected Area**	**Measured Area**	**Recovered (%)**	**Average Recovered (%)**
10	964729	994830	103.12	**101,48**
30	1656712	1657078	100.02	
50	2194398	2223191	101.31	

Table 3.9. Marein content in aqueous extract and ethyl acetate fraction using method 1. SD: Standard deviation; RSD: Relative standard deviation.

Sample	Average peak area	SD	%RSD	w/w dry extract (%)
Fresh aqueous extract **A**	2593512	57291.96	2.20	**4.28**
Fresh aqueous extract **B**	2366006	64079.38	2.71	**3.87**
AcOEt fraction **A**	2555406	92413.21	3.62	**16.84**

3.5. Quantification of selected compounds of *C. tinctoria* extracts by a different HPLC-UV method

As observed in section 3.3., *C. tinctoria* flowering tops aqueous extract and AcOEt fraction, are mainly constituted by chalcones and flavanones. Besides compound identification, compound stability and the quantification of multiple constituents are also important factors when addressing quality control of bioactive plant extracts.

In this study, due to the acidic conditions chosen for the HPLC separation procedure as well as sample conservation factors, interconversion of chalcone marein (**78**) into flavanone flavanomarein (**85**) and also into it's aurone, maritimein (**80**) was observed (Figure 3.6 A and B; Figure 3.9). Additionally, parallel *in vivo* and *in vitro* experiments (Chapter 4 and 5) appeared to indicate that more than one compound can be responsible for the observed bioactivity.

So, in the final stage of this phytochemical investigation, the content of some flavonoids was evaluated. In order to do so, a multiple-compound quantification method was developed (**Method 2**). The selected compounds were chalcone marein (**78**), flavanone flavanomarein (**85**) and two phenylpropanoic acids: chlorogenic acid (**28**) and diccaffeoylquinic acid (**97**). The experimental procedure was carried out by HPLC coupled to a DAD detector, as described in the experimental section (Chapter 8, section 8.6.2), and through which several samples of *C. tinctoria* flowering tops aqueous extract and AcOEt fraction, were analysed.

The content (in mg/L) of marein, flavanomarein and chlorogenic acid present in *C. tinctoria* extracts was calculated using the corresponding commercial standards. As for dicaffeoylquinic acid quantification, cynarin (1,3-dicaffeoylquinic acid) was used as standard. Standard curves were determined for individual standards taking into consideration a relationship between peak area field and standard concentration (Table 3.10). Measurements were made at the respective UV absorption maximum wavelengths: 280 nm for flavanomarein; 380 nm for marein and 320 nm for both chlorogenic and dicaffeoylquinic acids and linearity was determined in the range from 0.585 to 18.75 mg/L.

Quantification results, obtained using method 2, are displayed in table 3.11. For what was considered to be the major compound, marein, varied from 4.08 % (Sample D) to 4.60 % (Sample C) in the fresh aqueous extract and 4.26 % in the correspondent one-week old aqueous extract (Sample C). Considering the ethyl acetate fraction, marein content varied from 43% (Sample C) and 51% (Sample B).

On the other hand, flavanomarein and its diasterioisomer, were measured together and showed higher concentrations when comparing with marein, in all three aqueous extracts samples (varying between 4.4 to 7.9%).

The presence of a high content in flavanomarein (and isomer) is probably due to marein isomerization into flavanone structure that is favoured under acidic conditions used during the HPLC elution.

As previously described by Shikoriyama (1957) and Zhang (2006) chalcone marein and its corresponding flavanone, flavanomarein were found to dominate in *C. tinctoria* flowering tops while phenyl propanoic acids such as dicaffeoylquinic and chlorogenic acids were found in very low concentrations ≤1.5 %.

In order to understand marein concentration variations in *C. tinctoria* flowering tops aqueous extracts and AcOEt fractions, results from the measurements using methods 1 and 2 were compared (Table 3.12.).

Table 3.10. Standard curves equations.

Compound	Wavelength (nm)	Regression equation	Correlation coefficient
Marein	380	$y = 1300.1x + 153.3$	$R^2 = 0.9994$
Flavanomarein	280	$y = 526.2x + 132.6$	$R^2 = 0.9989$
Dicaffeoylquinic acid	320	$y = 1182.5 x + 137.6$	$R^2 = 0.9994$
Chlorogenic acid	320	$y = 926.3 x + 217.53$	$R^2 = 0.9990$

Table 3.11. Quantification of the four chosen metabolites present in several *C. tinctoria* flowering tops extracts and fractions using method 2.

	Percentage (w/w dry extract)			
Samples	**Marein**	**Flavanomarein** (both isomers)	**Chlorogenic Acid**	**Dicaffeoylquinic Acid**
Aqueous extract (fresh) C	4.60	7.14	0.59	0.35
Aqueous extract (old) C	4.26	7.91	1.31	0.80
Aqueous extract (fresh) D	4.08	4.44	0.30	0.32
AcOEt fraction B	51.43	20.29	1.24	3.60
AcOEt fraction C	43.14	40.19	n.d.	n.d.

n.d. = not detectable

Table 3.12. Marein quantification present in several *C. tinctoria* flowering tops extracts and fractions. Comparison between samples and quantification methods.

Samples	**Marein content**		
	Percentage (w/w dry extract)		
	Method 1	**Method 2**	**Average**
Aqueous extract (Fresh) A	4.28	-	4.218 ± 0.27
Aqueous extract (Fresh) B	3.87	-	
Aqueous extract (Fresh) C	-	4.60	
Aqueous extract (Old) C	-	4.26	
Aqueous extract (Fresh) D	-	4.08	
AcOEt fraction A	16.84	-	37.15 ± 18.04
AcOEt fraction B	-	51.43	
AcOEt fraction C	-	43.14	

From the samples analysed (Table 3.12) it is possible to observe that marein content in the aqueous extract is in average 4.22 % (w/w dry extract) of the dry extract, which was relatively constant using both methods.

Regarding the AcOEt fractions, using **method 1**, marein content was approximately 4 times more concentrated than in the aqueous extract. This information was important for subsequent dose determination for *in vivo* studies.

Later on in the course of this project, **method 2** was developed and, as expected, the results supported the previous determinations: AcOEt fraction was more concentrated in marein than the aqueous extract. The proportion of marein present in the fraction comparing with the aqueous extract was higher though, instead of 4 fold increase in marein content determined in method 1, using method 2, a nearly 11 fold increase was observed (comparing with the aqueous extract's marein content).

This high variability can result from a combination of factors that include not only different plant material but especially different preparation method of AcOEt fraction. This fraction was prepared by direct liquid-liquid fractioning from the freshly prepared (standard preparation) *C. tinctoria* flowering tops infusion until exhaustion. As reaching "exhaustion" was based in a qualitative evaluation (the disappearance of yellow colour in the solvent used for extraction), the volume of solvent was not controlled and therefore must have varied in each partition process. As a result, differently concentrated AcOEt fractions were obtained.

As the fractions were quantified prior to *in vivo* studies, the extrapolations relating the amount of marein and *C. tinctoria* extract's bioactivity were not compromised.

For optimal quantification results, for later standardization of *C. tinctoria* flowering tops extracts, a higher number of samples ought to be analysed, including plant material from different sources, followed by a fully controlled extraction and chromatographic separation procedures must be performed.

Overall, this study has successfully determined, for *C. tinctoria* flowering tops bioactive extracts, the optimal conditions for chromatographic separation, compound identification and quantification using a HPLC coupled with a diode array detector and a tandem mass spectrometer.