DENSITOMETRIC ASSAYS FOR THE EVALUATION OF WATER SOLUBLE ALKALOIDS FROM *CHELIDONIUM MAJUS* L. (PAPAVERACEA) ROOTS IN THE AZORES, ALONG ONE YEAR CYCLE

M. LEONOR PAVÃO & RUY E. PINTO

ARQUIPÉLAGO

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Every 4 weeks, during a one year cycle, water soluble alkaloid extracts were prepared from *Chelidonium majus*, L. (great celandine) roots, growing spontaneously on uncultivated ground in the Azores. TLC analysis showed that chelidonine, chelerythrine, sanguinarine, berberine, coptisine, protopine and allocryptopine are present in all the extracts. Fluorescence densitometry (366 nm) was found to be the most adequate densitometric technic to quantify chelidonine, berberine and coptisine. Sanguinarine and chelerythrine were analysed only by the fluorescence method. The use of the absorption mode for the evaluation of chelidonine, berberine and coptisine (at 242 nm, 344 nm and 268 nm, respectively) is also discussed. Chelidonine was the major constituent (about 70% of total quantifed alkaloids); maximum values appeared in July (60% higher than mean value) and minimum in January (60% lower). Both chelerythrine and sanguinarine concentrations showed maximum values in winter (about twice) and minimum in June (about one half). Berberine and coptisine exhibited relative small changes. Berberine and coptisine contents were maximum in July. The concentration of coptisine was minimum in December.

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Prepararam-se extractos de alcalóides solúveis em água de raízes de *Chelidonium majus* L. (celidónia), colhida em terrenos incultos nos Açores, mensalmente, ao longo de um ano. A separação dos alcalóides por cromatografia em camada fina mostrou que a quelidonona, queleritrina, sanguinarina, berberina, coptisina, protopina e alocriptopina são constituintes permanentes dos extractos. Utilizou-se a técnica de fluorescência (366 nm), julgado como o mais adequado, para a quantificação densitométrica da quelidonina, berberina e coptisina. A queleritrinae a sanguinarina só foram avaliadas por fluorescência. Nos casos da quelidonina, berberina e coptisina, é também discutida a utlização do modo de absorção (a 242 nm, 344 nm e 268 nm, respectivamente). A quelidonina revelou ser o constituinte principal (cerca de 70% do total dos alcalóides avaliados) em todos os extractos; os valores máximos da sua concentração surgiram em Julho (60% mais alto do que o valor médio) e o mínimo em Janeiro (60% mais baixo). As concentrações da queleritrina e da sanguinarina foram mais elevadas no inverno (cerca do dobro) e mínimas em Junho (cerca de metade). A berberina e a coptisina mostraram variações de concentração relativamente pequenas. As concentrações de ambas foram máximas em Julho, sendo a da coptisina mínima em Dezembro.

M. Leonor Pavão, Departamento de Ciências Tecnológicas e Desenvolvimento, Universidade dos Açores, R. Mãe de Deus, PT-9500 Ponta Delgada, Açores, Portugal. - Ruy E. Pinto, Departamento de Química e Bioquímica, Faculdade de Ciências, Universidade de Lisboa, R. Ernesto Vasconcelos, Bloco C1, 5° Piso, PT-1700 Lisboa, Portugal.

INTRODUCTION

Chelidonium majus L. (great celandine) is a medicinal plant known since immemorial times in Eastern and Central Europe and in some regions of Asia and America. Many alkaloids (at least 25) have been identified from the latex of this Papaveracea. However, the alkaloid identity and contents change with climate, organ and developmental stage of the plant (TERNBAH 1958; KIM et al. 1969; KUSTRAK et al. 1982). Also, it has been difficult to obtain both a satisfactory separation and estimation of the nitrogen bases, due to the similarity of their chemical structures (for a revision see SEQUEIRA DE MEDEIROS 1984).

In Portugal, the plant is mainly used in the Azores islands, for the properties of its latex (PEREIRA 1953). As we are interested in our own plants and their biochemical effects in mammals (SEQUEIRA DE MEDEIROS 1990), it is important to choose the best harvesting time in the Azores. Because no work about alkaloids in this plant has been reported in Portugal, we decided to carry out the present study. The purpose is to examine the variations in the major components in water soluble alkaloid extracts (WSA) prepared from the roots of *Chelidonium majus*, during a single growing season, by assaying a new quantitative densitometric methodology.

Separations of the alkaloid fractions were done by TLC, using three solvent systems. Fluorescence and absorption densitometric assays were carried out to evaluate the identified benzophenanthrydine and protoberberine bases: chelidonine, chelerythrine, sanguinarine, coptisine, berberine, protopine and allocryptopine.

MATERIALS AND METHODS

Plant

Plants were harvested every 4 weeks, during a one year cycle, on uncultivated ground in the island of S. Miguel. Roots (3-4 kg) were separated from the aerial parts and dried at 50-60°C for 3 days. The dried material (tissues from all the collected plants combined together) was coarsely powdered.

Chemicals

All chemicals were of analytical grade. Berberine chloride ($2H_2O$), chelidonine and sanguinarine were from Extrasynthèse®, 69000 Geenay, France. Chelerythrine, protopine, allocryptopine and coptisine were kindly provided by Professor Slavik (Purkine University, Brno, Czech Rep.).

Water soluble alkaloid extracts

The determination of total alkaloids from roots was carried out according to KUSTRAK et al. (1982), except that the extract was refluxed for 1/2 hour and let stand at least 24h at room temperature (LAVENIR & PARIS 1965). All the residues were dried under N_2.

One part of powdered material was refluxed for 1/2 hour with sixfold volume of methanol. The solvent was removed by distillation (80°C) and the residue fully dried at 100°C under N_2. The dry methanolic residue was extracted by several work-ups with portions of 3N sulfuric acid until the decanted liquid gave a negative Mayer test. The acidic extracts were combined, made alkaline (pH9) with concentrated aqueous ammonia, and the liberated alkaloids shaken out with chlorophorm. The separated chlorophorm layer was freed of solvent by distillation and the residue was dried as before.

WSA were obtained by adding sucessive small portions of hot distilled water to the alkaloid concentrate, until no yellow colour was observed. Then, the solutions were evaporated until dry and weighed.

TLC procedure

Spotting samples were prepared by dissolving the dry WSA in methanol (1 mgWSA/ml). Samples of 10 and 20 µl were applied on ready made 5x20cm glass plates, covered with a 0.25 thick silica gel-60 G-F_{254} layer (Merck®).

Chelidonine, chelerythrine, sanguinarine, berberine, coptisine, protopine and allocryptopine in methanolic solutions (concentrations between 1 and 10mM, according to the respective fluorescence intensity) were used as comparison standards.

Three developing systems (S_1, S_2 and S_3) were selected for the TLC procedure (KUSTRAK et al. 1982). The compositions of S_1, S_2 and S_3 are the following:

- xylene- methylethylketone-methanol-diethylamine 20:20:2:0.5 (vol)
- chloroform-ethil acetate-methanol 30:45:25 (vol)
- ciclohexane-diethylamine 85:15 (vol)

Each eluent was freshly prepared before being poured into the tank, which walls had been previously covered with Whatman 3M filter paper.

Unidirection developments of 16 cm were allowed at room temperarature. The chromatograms were scrutinized under UV irradiation (366 nm), using a Camag lamp; for initial confirmation tests, some of them were also sprayed with the Dragendorff reagent.

A relatively large number of alkaloid fractions was observed. However, only those for which we had standards were identified, by using the R_f comparison between the standards and the unknown alkaloids. TLC procedures and the respective densitometric analysis were always performed in the same day.

Densitometric analysis

All the densitometric estimations were performed by a TLC Scanner II (Camag), provided with a fluorescence detector and connected to an integrator Hewlett-Packard, model 3392A. A 10 nm thick beam from the mercury lamp was selected for all scannings.

Each of the following alkaloids was evaluated from the chromatograms where a better resolution was observed: chelidonine was quantified from S_1, coptisine from S_2 and berberine from S_3 chromatograms, by fluorescence and by absorption densitometry. Chelerythrine and sanguinarine (from S_1 chromatograms) were evaluated only by the fluorescence mode. In fact, both the R_f values and the absorptive λ_{max} are too close together to permit resolution using the absorptive method.

In all cases, given the presence of unknown fractions, a horizontal scanning was performed, along the spots with the same R_f on different runs of the same sample.

The run speed of the plates in the densitometer was programmed to be 1 mm/s and the integrator paper sheet speed was 4 mm/s.

All the fluorescence determinations were carried out by using a K400 filter and a 366 nm exciting irradiation on the selected spots. Calibration curves were used to calculate, for each alkaloid, the standard applied amount responsible for each "densitometer unit" (Table 1). Also, different volumes of methanolic solutions, with concentrations between 0.02 and 0.04 mg of WSA/ml were applied and proportionality between concentrations and respective densitogram area was observed.

For the absorption evaluations, each alkaloid analysis wavelength was selected from the respective UV/VIS spectrum (absorptive λ_{max} of the alkaloid in aqueous solution): 242 nm for chelidonine, 344 nm for berberine and 268 nm for coptisine (SEQUEIRA DE MEDEIROS 1990). The procedure was the same as described for fluorescence (Table 1).

RESULTS AND DISCUSSION

TLC analysis

For the best resolution, solvent S_1 allowed the separation of 14 alkaloid fractions; however, only the alkaloids that could be compared with reference standards were identified (Table 2). S_1 showed to be the best solvent system for evaluating the benzo[C] phenanthrydine bases, when in low concentrations. Besides, it was the

only one that revealed the presence of allocryptopine, wich is not fluorescent at 366 nm; when using S_2, allocryptopine was partially mixed with berberine and overlapped with chelidonine in S_3 chromatograms.

S_2 was particulary useful to detect protopine by fluorescence, since no such behaviour was observed for this alkaloid, when using the other two developing systems. However, its resolution was not good enough to quantify it. S_2 also allowed a better resolution of berberine and coptisine than S_1, but was unable to make the distinction among the three benzo[C] phenanthridine bases.

S_3 was the best solvent for the resolution of chelidonine, chelerythrine and sanguinarine, when no other alkaloids were present. In fact, when using S_3 for the development of WSA samples, chelidonine and allocryptopine have identical R_f and the same occurred with coptisine and chelerythrine. Sanguinarine was poorly detected with S_3. X_4 was recognized as one of the main, but not identified, fluorescent alkaloids in all the extracts.

Table 1

Concentration of standard alkaloids (per "densitometer unit"). Values represent mean±sd (6 samples)

Solvent	Alkaloid	Mode/l (nm)	Conc. (pg)
1	Chelidonine	Fl./366	5.6±0.02
		Abs./242	3.2±0.02
	Chelerythrine	Fl./366	4.7± 0.04
		Abs./268	0.63± 0.001
	Sanguinarine	Fl./366	14.4± 0.05
		Abs./276	1.0± 0.5
2	Coptisine	Fl./366	0.26± 0.001
		Abs./268	0.27± 0.007
	Protopine	Fl./366	94.2± 0.4
		Abs./288	11.1± 0.06
3	Berberine	Fl./366	0.06± 0.004
		Abs./344	0.05± 0.002

Fl. = Fluorescence; Abs. = Absorption.

Table 2

WSA composition patterns by $TLCR_f$ may be considered as typical values (error <10%, for a large number of applications)

S	Alkaloid	Rf	Fluoresc. (366 nm)	Colour (Dragendorff)
	C	0	bright yellow	brown
	B	0.02	bright green	violet
	X1	0.09	yellow	—
	X2	0.12	greenish yellow	—
	A	0.19	—	dark yellow
1	P	0.38	—	brownish yellow
	X3	0.39	light blue	—
	X4	0.46	pink	yellow
	X5	0.53	light pink	—
	X6	0.62	violet	—
	X7	0.66	pink	yellow
	Ch	0.69	yellow	yellow
	Chr	0.71	bright yellow	yellow
	S	0.75	red	light pink
	C	0.02	bright yellow	brown
	B+X5	0.03	bright green	violet
	A	0.05	—	dark yellow
2	P	0.12	blue	brownish yellow
	Y1	0.17	light blue	—
	Y2	0.28	light pink	—
	X4	0.31	pink	yellow
	Ch+Chr+S	0.66	yellow	orange
	X5	0.03	light pink	—
	Z1	0.09	violet blue	—
	Z2	0.12	yellow	yellow
3	Ch+A	0.19	yellow	yellow
	X4+P	0.26	yellow	dark yellow
	B	0.37	bright green	violet
	C+Chr	0.41	yellow	dark yellow
	S	0.5	pink	light pink

S_1=xylene- methylethylketone- methanol-diethyl-amine 20:20:2:0.5 (vol); S_2= chloroform-ethil acetate-methanol 30:45:25 (vol); S_3=ciclohexane-diethylamine 85:15 (vol); Ch=chelidonine; Chr= chelerythrine; S=sanguinarine; B=berberine; C= coptisine; P= protopine;A= allocryptopine.; X_1-X_7, Y_1, Y_2, Z_1-Z_5=not identified alkaloid fractions.

Densitometric evaluation

Considering the described criteria for the densitometric analysis of the assayed alkaloids, the fluorescence method should be more reliable than the absorptive one. In fact, it takes advantage of two locating parameters (R_f and fluorescence colour at 366 nm). On the other hand, the selected wavelengths for the absorption analysis may not correspond exactly to the absorptive λ_{max} of the alkaloid derivates on the chromatograms. However, a reasonable agreement was found between fluorescence and absorption data obtained for chelidonine, berberine and coptisine standards, but not for sanguinarine and chelerythrine (Table 1).

No relevant differences were registered for chelidonine and berberine concentrations in WSA by using both methods. For coptisine, the values obtained by absorption were always much higher (at least twice) than the respective fluorescence data (Table 3). The fluorescent alkaloids that were estimated represent about one half of the total WS alkaloids.

Table 3

Concentration of alkaloids from water soluble extracts of Chelidonium roots, along a one year cycle. Values represent mean ± sd (3 samples).

Month	Mode	Alkaloid Concentration Ch	Chr	S	B	C
Feb.	Fl.	227.9± 5.9	35.5± 1.7	38.6± 0.4	25.5± 0.1	21.2± 0.1
	Abs.	281.4± 6.1	-	-	21.9± 0.7	50.4± 0.0
March	Fl.	301.9± 1.0	39.9± 0.6	25.6± 0.4	16.4± 0.1	17.7± 0.0
	Abs.	325.5± 5.5	-	-	16.2± 0.1	47.2± 0.5
April	Fl.	213.3±0.5	22.1± 1.0	28.9 ± 0.6	16.3± 0.1	18.9± 0.1
	Abs.	220.1± 4.3	-	-	18.4± 0.1	61.2± 0.2
May	Fl.	291.0± 11.0	29.2± 0.2	37.7± 1.0	24.8± 0.2	31.9± 0.8
	Abs.	293.5± 2.6	-	-	25.4± 0.1	81.8± 0.9
June	Fl.	198.3± 4.3	19.3± 0.2	12.5± 1.4	11.6 ± 0.0	16.0± 0.3
	Abs.	219.9± 2.0	-	-	11.0± 0.1	65.5± 1.8
July	Fl.	317.4± 0.8	27.4± 0.6	31.4± 0.2	31.2± 0.2	35.5± 0.9
	Abs.	332.9± 1.7	-	-	27.5± 0.2	77.7± 1.2
Sept.	Fl.	233.9± 0.3	21.6± 0.3	40.5± 1.5	17.4± 0.4	15.0± 0.1
	Abs.	276.2± 12.5	-	-	16.4± 0.2	37.1± 0.1
Oct.	Fl.	297.0± 17.9	30.4± 0.8	54.5± 0.9	21.8± 0.1	15.2± 0.1
	Abs.	293.7± 15.9	-	-	18.5± 0.0	50.9± 0.5
Nov.	Fl.	216.6± 1.9	25.9± 0.2	61.9± 0.4	20.4± 0.1	15.6± 0.1
	Abs.	298.8± 1.0	-	-	16.8± 0.4	40.0± 0.4
Dec.	Fl.	204.7± 8.4	60.0± 0.2	69.1± 9.9	24.5± 0.2	13.3± 0.0
	Abs.	198.2± 1.7	-	-	20.6± 1.3	51.1± 0.2
Jan.	Fl.	138.0± 8.5	42.9± 0.2	67.0±2.2	23.9± 0.1	22.1± 0.1
	Abs.	145.7± 7.4	-	-	24.8± 0.4	54.1± 0.2

Month=Month of roots harvesting for preparation of extracts; Fl.=Fluorescence; Abs.=Absorption; Ch=Chelidonine; Chr=Chelerythrine; S=Sanguinarine; B=Berberine; C=Coptisine.

Chelidonine, chelerythrine, sanguinarine, berberine, coptisine, protopine, allocryptopine and X_4 appeared to be permanent constituents of the latex of *Chelidonium* roots in S. Miguel during the entire year. Chelidonine is the major alkaloid (about 70% of the quantified alkaloids). Maximum values appeared in July (60% higher than the mean value) and minimum in January (60% lower). Both chelerytrine and sanguinarine concentrations reached maximum values in winter (about twice the mean value) and minimum in June (about one half). Berberine and coptisine concentrations exhibited relatively small changes along the year. They were maximum in July and minimum in December.

It was not possible to identify X_4; however, we suggest it is α- homochelidonine. In fact, this alkaloid and chelidonine have similar chemical structures and properties (SANTAVY et al. 1960). Besides, α- homochelidonine is one of the main components of the root latex of the plant (SEOANE 1965; KIM et al. 1969; KUSTRAK et al.

1982), which seems to agree with the present observations.

A possible coptisine and berberine overlap can not explain the higher absorptive values obtained for coptisine, because both berberine concentration and absorptivity were lower than those of coptisine. Thus, the present work suggests that some other alkaloid fraction, with about the same R_f as coptisine (not fluorescent but absorbing at 268 nm) may exist in all the extracts. Its concentration changes along the year, reaching maximum values between January and July and minimum between July and September.

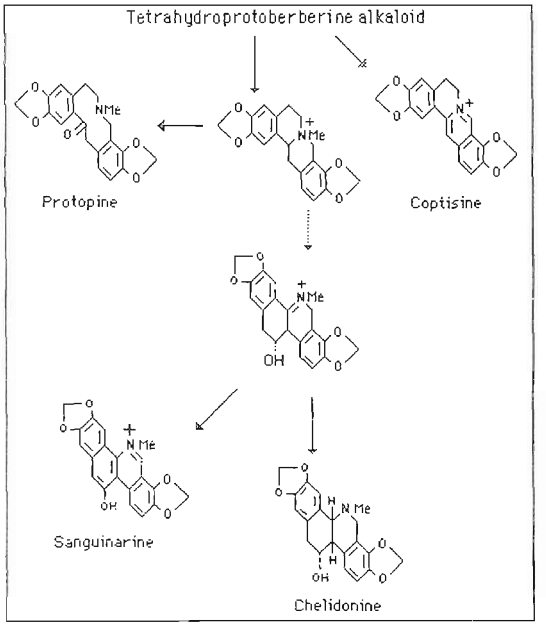

Figure 1. Biosynthetic relationshipis among some alkaloids of *Chelidonium majus* (adapted from BATTERSBY et al. 1975).

According to BATTERSBY et al. (1975) benzophenanthridine alkaloids arise by a modification of the tetrahydroprotoberberine skeleton. Namely, chelidonine and sanguinarine (chelerythrine has the same aromatic system) are formed from a common intermediate (Fig. 1). This agrees with our findings that concentration of chelidonine is higher when sanguinarine and chelerythrine are lower.

TLC with solvents 1 and 2 followed by the described densitometric quantitative analysis is a reliable method to quantify chelidonine and berberine in WSA from *Chelidonium majus*. Solvent 1 also permited evaluation of sanguinarine and chelerythrine by the fluorescence method. However, results should be confirmed using other techniques.

Concerning coptisine, a new solvent system should be assayed to confirm our suggestion that this alkaloid overlaps with a non-fluorescent unknown.

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