Metabolite fingerprinting of *Camptotheca acuminata* and the HPLC–ESI-MS/MS analysis of camptothecin and related alkaloids

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A B S T R A C T

The major phytochemical constituents, namely, alkaloids, flavonoids and ellagic acid derivatives, of leaves of *Camptotheca acuminata* were identified using high performance liquid chromatography (HPLC) coupled with electrospray mass spectrometry (ESI-MS) in extracts of plants cultivated in Italy and collected at different growth stages. Alkaloids related to camptothecin were identified and quantified by HPLC coupled with ESI-tandem mass spectrometry (MS/MS) employing, respectively, an ion trap and a triple quadrupole mass analyser. The fragmentation patterns of alkaloids related to camptothecin were analysed and a specific Multiple Reaction Monitoring HPLC–MS/MS method was developed for the quantitative determination of these constituents. The described method provides high sensitivity and specificity for the characterisation and quantitative determination of the alkaloids in *C. acuminata*.

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1. Introduction

Camptothecin (CPT), an important constituent of a number of species of the genus *Camptotheca*, is regarded structurally as a quinoline alkaloid but, on a biogenetic basis, should be classified as a modified monoterpenoid indole alkaloid. CPT was originally shown to exhibit antineoplastic activity in the early 1960s [1], and the alkaloid and its analogues were the first compounds reported to stop cell division by directly blocking the DNA replication enzyme topoisomerase I [2]. Although CPT has shown potential as an anti-cancer drug, the low solubility of the alkaloid limits its use in therapy. In this regard, the naturally occurring 10-hydroxycamptothecin and the semi-synthetic water-soluble derivatives topotecan and irinotecan are more promising. The latter two derivatives have already been approved by the Federal Drug Administration (FDA) for the treatment of ovarian, lung and colorectal cancer, and 9-nitrocamptothecin is expected to receive FDA approval for pancreatic cancer therapy in the near future. Additionally, a number of other CPT derivatives have shown promising results in clinical trials.

Although the total synthesis of CPT is technically possible [3,4], extraction of the alkaloid from natural sources is considerably more cost effective. In this respect, CPT can be readily extracted from the bark and stems of *Camptotheca acuminata* Decne. (Cor-naceae), a rapidly growing tree that can attain an average height of 10–15 m. Not only is this species a valuable source of CPT for the semi-synthesis of bioactive molecules, but it also accumulates hydroxycamptothecin. This CPT derivative has been shown to be more potent and less toxic than CPT [5] and exhibits a strong apoptosis-inducing effect on human hepatoma Hep G2 cells [6,7]. Other CPT derivatives are present in *C. acuminata*, but few pharmacological studies have been conducted on these components.

It is presumed that CPT is one of the defence compounds of *C. acuminata* [8], and that the co-occurring derivatives are formed by interconversion reactions, possibly induced in response to changes in environmental conditions. If this assumption is true, then it is important to determine the pattern of accumulation of CPT derivatives and their organ specificity. A number of studies have focussed on the accumulation of secondary metabolites, especially quinoline alkaloids, ellagic acid derivatives and flavonoids, and their variation in different parts of *C. acuminata* of diverse origin and in the early phases of plant development [9,10].

Several approaches have been employed for the analysis of CPT, mainly involving chromatographic purification followed by physicochemical analysis by mass spectrometry (MS), nuclear magnetic resonance (NMR), etc. Additionally, a number of reports are available concerning the analysis of CPT in *C. acuminata* samples by high performance liquid chromatography (HPLC) [11–13]. Recently, alternative simple and sensitive methods involving HPLC coupled with mass selective detection have been improved [14], and these have been shown to be more accurate than HPLC-UV.

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HPLC coupled with an atmospheric pressure ionisation source is the method of choice for the analyses of highly polar compounds present in infusions [15,16]. Electrospray ionisation (ESI) is currently a very popular technique since it produces a soft ionisation that can lead to the formation of protonated or deprotonated molecular ions [17]. Tandem mass spectrometry (MS/MS) provides further information about the structure of an analyte since it is possible to fragment the parent ion and to obtain structurally important daughter ions [18]. Thus, HPLC on-line with ESI-ion trap (IT)-MS provides a fast method to separate and determine the molecular mass of a compound as it elutes directly from the chromatographic column, while HPLC–ESI-triple quadrupole (QqQ)-MS allows the quantification of metabolites using a very sensitive and selective mass tandem experiment such as Multiple Reaction Monitoring (MRM).

HPLC–MS/MS has been applied to the analysis of SN-38 (a synthetic derivative of CPT) in rat plasma using CPT as internal standard together with an MRM method focused on the loss of a neutral unit of 44 amu [19]. The same method has also been adapted to the simultaneous analysis of CPT-11 and SN-38 in mouse plasma and tissues [20].

The aim of the present study was to develop and validate an HPLC–ESI-IT-MS/MS method for the qualitative fingerprinting (metabolic profiling) of tissues of *C. acuminata* with respect to three different classes of compounds present in the extracts, namely, alkaloids (CPT and its co-occurring derivatives), ellagic acid derivatives and phenolic compounds. An additional objective was to develop and validate an HPLC–ESI-QqQ-MS/MS method by which to quantify CPT and co-occurring related compounds in different organs of *Camptotheca* plants cultivated under controlled agronomic conditions in Italy. On the basis of these studies, it was possible to propose a mechanism for the observed fragmentation of CPT and its derivatives under the MS conditions employed.

2. Materials and methods

2.1. Chemicals

Solvents used for extraction were of high purity and purchased from Carlo Erba (Milano, Italy). HPLC grade methanol, acetonitrile and trifluoroacetic acid were purchased from J.T. Baker (Baker Mallinckrodt, Phillipsburg, NJ, USA). HPLC grade water (18 mΩ) was prepared using a Millipore (Bedford, MA, USA) Milli-Q purification system. CPT standard was purchased from Extrasynthese (Geney, France) and methyl 4-methoxy-2-indolecarboxylate standard was purchased from Sigma–Aldrich (Poole, UK). All other chemicals used in this study were of analytical grade.

2.2. Plant material and the preparation of extracts

Specimens of *C. acuminata* were cultivated in an experimental field near the coastal area of Pisa (Tuscany, Italy) in the spring 2002 by the Department of Agronomy and Agro Systems of Pisa University. The plant of *C. acuminata* spent the winter in pots in a greenhouse cold until spring 2003. In May plants were transplanted in the field at the experimental farm of Rottaia in San Piero a Grado (Pi) near to the Department of Agronomy and Agro Systems of Pisa University. Field was on a plot of sandy-texture (sand 59.97%, silt 33.79%, clay 6.4%) 1.43% organic matter, total nitrogen 0.77 g kg^{-1}; phosphorus (Olsen method) 4.90 mg kg^{-1}; potassium (Dirks Schepper method) 183.58 mg kg^{-1}; CaCO$_3$ 1.44%, pH 8.19 with a rather superficial ground water that never goes below 120 cm. The fertilisation was carried out with the administration of 100 kg ha^{-1} nitrogen (urea), 100 kg ha^{-1} phosphorus, and 100 kg ha^{-1} potassium (potassium sulphate). After the transplant was carried out

supplementary irrigation to encourage the emergence of plants, in addition to irrigation made throughout cultivation period. *C. acuminata* showed a good ability to adapt to the crop.

Following different periods of plant development (i.e. 3 or 4 years), samples of aerial organs were harvested at different times of the year, and were stored after air or freeze drying. The samples studied were: freeze-dried leaves harvested in August 2003 and in August 2004; freeze-dried leaf buds collected in August 2004; and air-dried leaves collected on 1st September 2004 and on 13th October 2004. In each case, 1 g of plant material was extracted with 10 mL of ethanol:water (7:3) with ultrasound agitation for 10 min, and then stored in the dark overnight. Samples were filtered and subsequently diluted 1:100 with ethanol:water (7:3) prior to analysis.

Prof Mario Macchia (Department of Agronomy and Agro Systems of Pisa University), authenticated the specimens, and a sample voucher of leaves and buds is stored at the Department of Agronomy and Agro Systems of Pisa University, voucher number 38.

2.3. HPLC-UV analyses

An Agilent (Palo Alto, CA, USA) 1100 series chromatographic system, comprising a G-1312 binary pump, a G-1328A Rheodyne injector (20 μL injection loop), a G-1322A degasser and a G-1315A photodiode array detector, was employed. Analyses were carried out using a Waters (Milford, MA, USA) XTerra C18 column (150 mm × 2.0 mm i.d.; 5 μm particle size) eluted with mixtures of water containing 0.05% trifluoroacetic acid (TFA; solvent A) and acetonitrile containing 0.05% TFA (solvent B) at a flow rate of 0.25 mL/min. Elution was with a gradient commencing at 100% A and changing to 90:10 (A:B) in 10 min, then from 90:10 (A:B) to 70:30 (A:B) in 20 min, then from 70:30 (A:B) to 50:50 (A:B) in 10 min, and finally to 100% B in 10 min. Detection was at 254 nm for alkaloids related to CPT and at 320 nm for phenolic compounds. Under these chromatographic conditions the alkaloid and phenolic constituents were well separated.

2.4. ESI-MS and ESI-MS/MS analyses

Full scan ESI-MS and collision induced dissociation (CID) ESI-MS/MS analyses of standard CPT were performed on a Thermo Electron (San Josè, CA, USA) LCQ Deca IT spectrometer equipped with an ion trap analyser. The analytical parameters were optimised by infusing a standard solution of CPT (1 μg/mL in methanol) into the source at a flow rate of 5 μL/min. The capillary voltage was 5 V, the spray voltage was 5 kV, the tube lens offset was 35 V, and the capillary temperature was 220 °C. Data were acquired in the positive ion MS and MS/MS modes.

2.5. HPLC–ESI-MS and HPLC–ESI-MS/MS analyses

Qualitative on-line HPLC–ESI-MS analyses of extracts were performed using a Thermo Finnigan (Thermo Electron) Spectra System HPLC coupled to a Thermo Electron LCQ Deca IT spectrometer. The chromatographic conditions were as described above for HPLC-UV analyses. The flow from the chromatograph was injected directly into the ESI source, maintained at a temperature of 280 °C, and MS were measured under the optimised parameters indicated for the ESI-MS and ESI-MS/MS analyses with nitrogen supplied at a flow rate of 80 (arbitrary units). MS data were acquired using the software provided by the manufacturer, and reconstructed ion chromatograms (RICs) were elaborated in order to identify alkaloids and flavonoids from their protonated molecular ions. In HPLC–ESI-MS/MS experiments, data were acquired using the dependent scanning mode in which the MS software chose, in real time, which ion to fragment on the basis of charge and intensity,
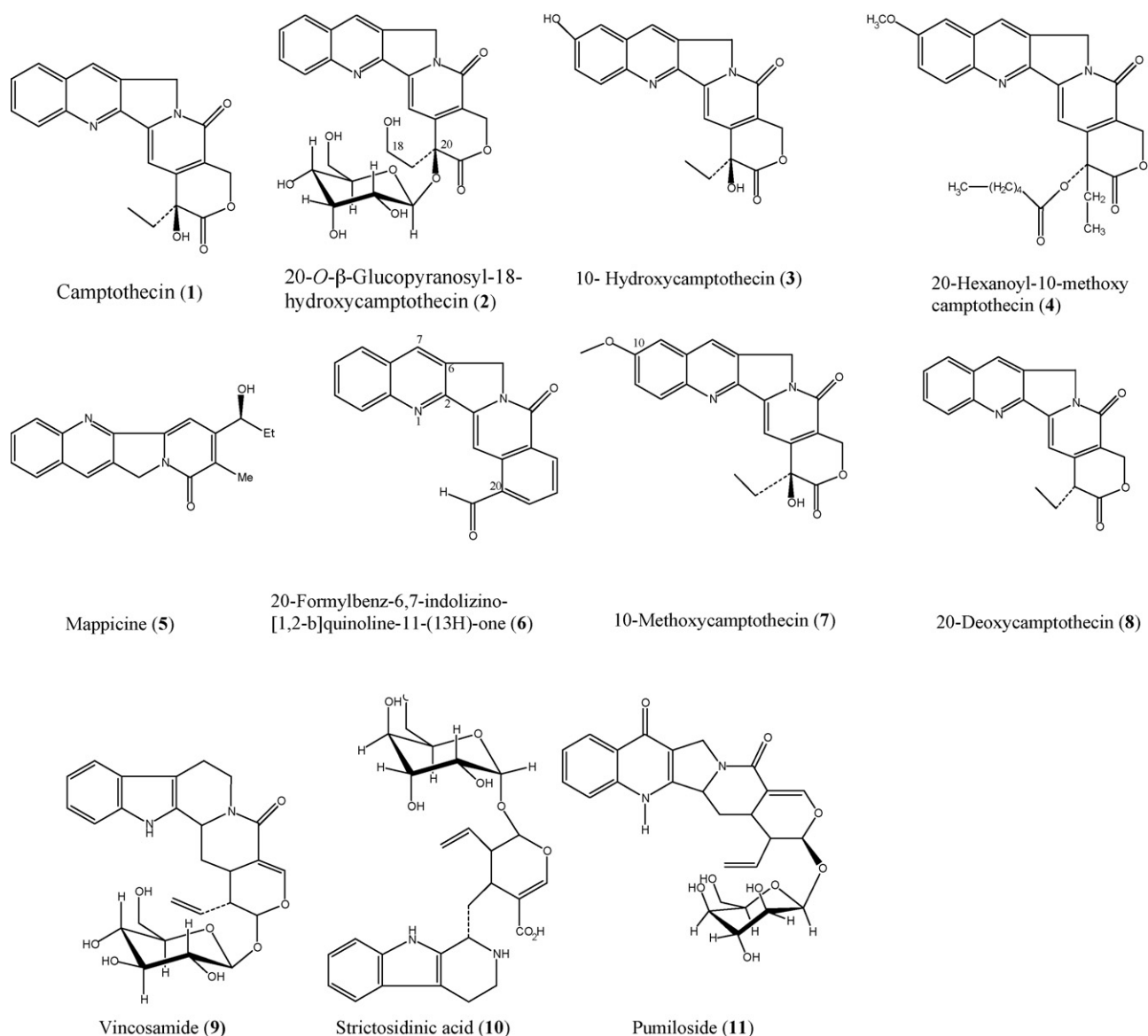
Fig. 1. Alkaloids identified in extracts of leaves and leaf buds of *Camptotheca acuminata*.

and optimised all parameters in order to improve ionisation and fragmentation.

Quantitative on-line HPLC-ESI-MS/MS analyses of the extracts were performed using an Agilent 1100 HPLC system interfaced to a Applied Biosystems (Foster City, CA, USA) API2000 instrument. The chromatographic conditions were as described above for HPLC-UV analyses. The API 2000 ES source was tuned by infusing a standard solution of CPT (1 μg/mL in methanol) into the source at a flow rate of 10 μL/min. The optimised parameters were: declustering potential 100 eV, focusing potential 170 eV, entrance potential 10 eV, collision energy 50 eV, and collision cell exit potential 5 eV. The spectrometer was used in the MS/MS mode with MRM of fragmentation reactions selected for each alkaloid as described below.

2.6. Calibration and quantification of CPT

In order to prepare the calibration plot, a sample (10 mg) of standard CPT was weighed accurately into a 10 mL volumetric flask, dissolved in methanol and the volume made up to the mark with methanol. The resulting stock solution was diluted with methanol in order to obtain reference solutions containing 5, 10, 20, 40 and 60 μg/mL of external standard. An appropriate amount of the internal standard (IS; methyl 4-methoxy-2-indolecarboxylate) was added to each reference CPT solution to give a final concentration of 10 μg/mL. Calibration curves were constructed by analysing reference CPT/IS solutions in triplicate at each concentration level. The ratios of the peak areas of the external standard to those of the IS were calculated and plotted against the corresponding standard concentration using weighted linear regression to generate standard curves. All quantitative data were elaborated with the aid of Analyst software (Applied Biosystems).

3. Results and discussion

The alkaloids and phenolic compounds identified in leaves of *C. acuminata* leaves are presented in Figs. 1 and 2, respectively.

3.1. Qualitative HPLC–ESI-IT-MS and HPLC–ES-IT-MS/MS analyses of C. acuminata extracts

The samples were initially analysed by HPLC-UV using two different monitoring wavelengths, namely, 254 nm for alkaloids
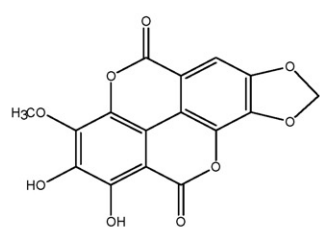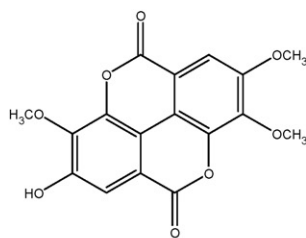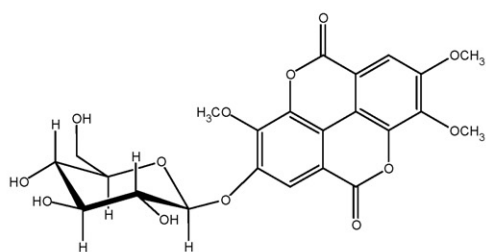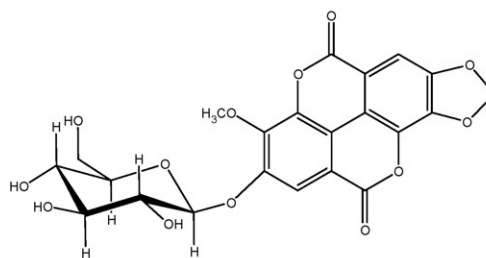
3,4-Methylendioxy-3'-*O*-methyl-5'-hydroxyellagic acid (**12**)

3,3',4-Tri-*O*-methylellagic acid (**13**)

3,3',4-Tri-*O*-methylellagic acid-4'-*O*-D-glucopyranoside (**14**)

3,4-Methylendioxy-3'-*O*-methyl-4-glucopyranosyl ellagic acid (**15**)
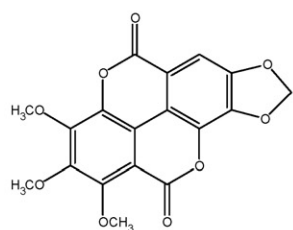
3,4-Methylenedioxy-3',4'-*O*-dimethyl-5'-methoxyellagic acid (**16**)
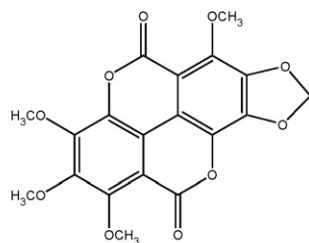
3,4-Methylenedioxy-3',4'-*O*-dimethyl-5,5'-dimethoxyellagic acid (**17**)
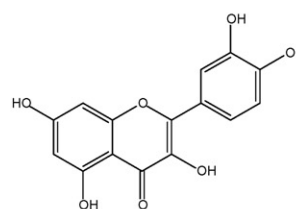
Quercetin (**18**)
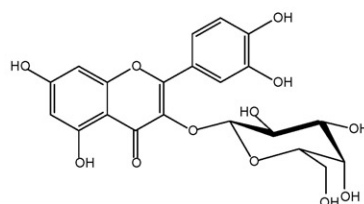
Quercetin 3-*O*-D-galactopyranoside (**19**)
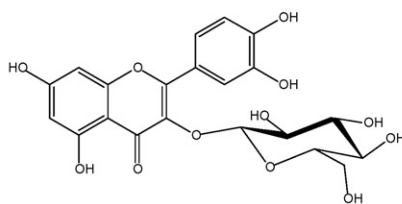
Quercetin 3-*O*-D-glucopyranoside (**20**)
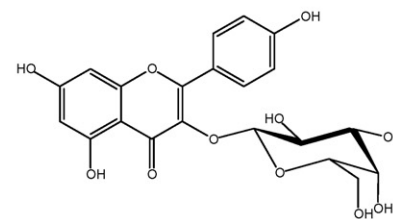
Kaempferol 3-*O*-D-galactopyranoside (**21**)
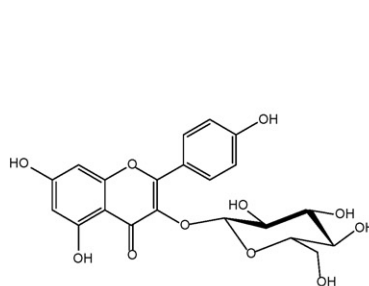
Kaempferol 3-*O*-D-glucopyranoside (**22**)
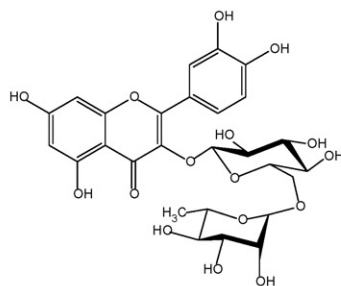
Rutin (**23**)
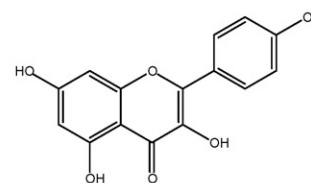
Kaempferol (**24**)

Fig. 2. Phenolic compounds identified in extracts of leaves and leaf buds of *C. acuminata*.

related to CPT and 320 nm for phenolic compounds. These preliminary studies were important in order to optimise the HPLC conditions based on the use of a Waters XTerra C18 column gradient eluted with a mobile phase comprising water and acetonitrile each containing 0.05% TFA. The chromatographic method developed allowed the separation of alkaloids, flavonoids and phenolic compounds related to gallic acid in a single chromatographic run.
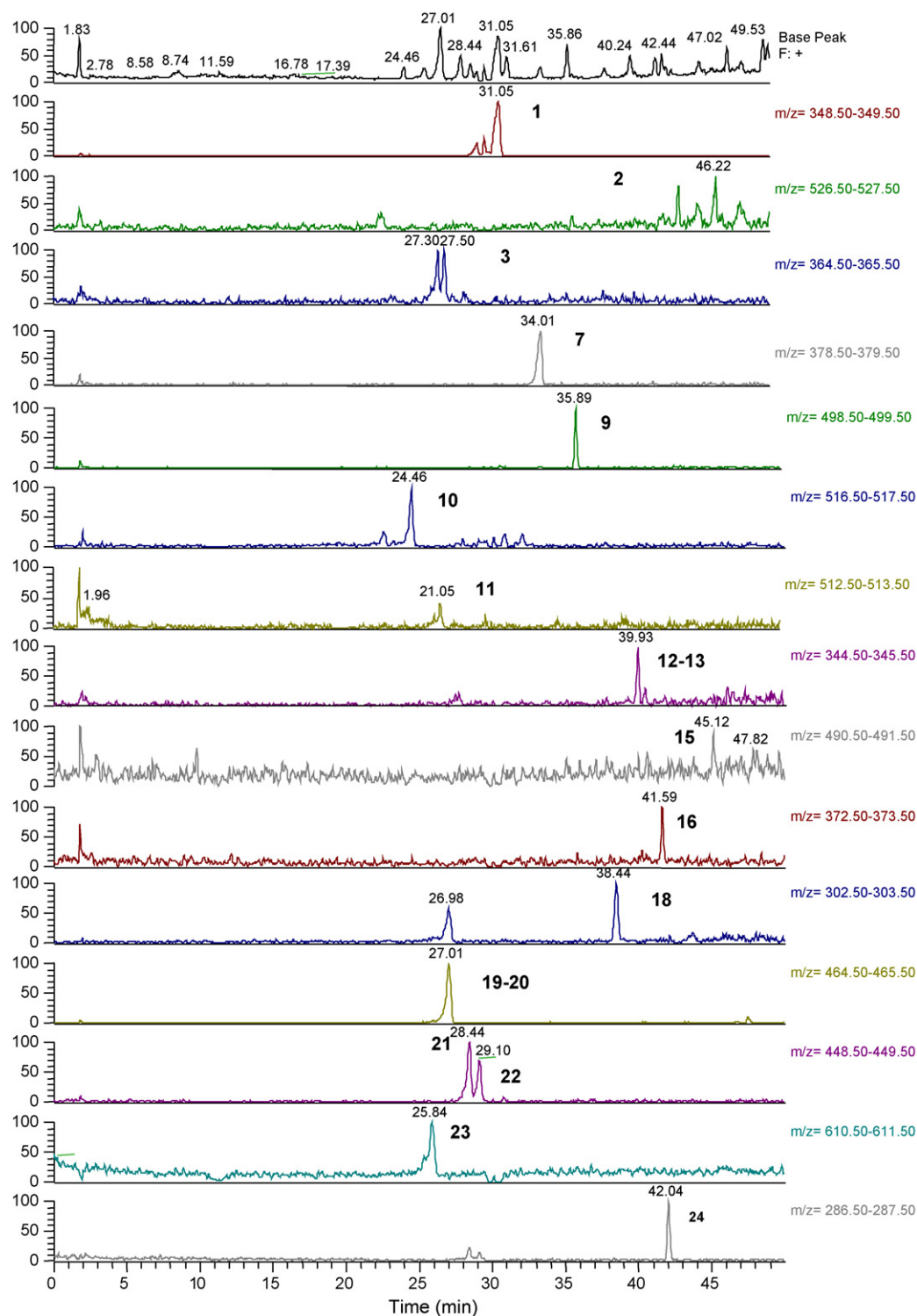
Fig. 3. Qualitative metabolite fingerprint of an extract of dried leaves of *C. acuminata* showing the TIC profile (top panel) and various RICs (lower panels) obtained from positive ion mode HPLC–ESI-IT-MS analysis.

HPLC–MS analyses were carried out in the positive ion mode over the *m/z* range (280–700) encompassing the protonated molecular ions of all constituents of *C. acuminata* described in the literature. A full MS scan, in the form of a total ion current (TIC) chromatogram, was initially acquired, following which RICs were derived for each of the expected *m/z* values based on the molecular weights of the possible constituents. This step was very important in order to attain a higher selectivity for the quantitative analysis.

Through the application of such HPLC/MS experiments, a large group of compounds could be identified in the extracts without time-consuming pre-purification steps or full optimisation of the chromatographic procedures. Fig. 3 shows the TIC profile and various RICs obtained from the positive ion mode HPLC–ESI-IT-MS analysis of an extract of dried leaves of *C. acuminata* collected on 13th October 2004. As may be observed, the majority of compounds were efficiently separated, and it was possible to recognise peaks

Table 1
Chromatographic and spectroscopic data for compounds **1**–**24**.

	t_R (min)	Compound	[M+H]$^+$ (m/z)	HPLC–ESI-MS^n (m/z)
1	31.04	Camptothecin	349	MS^2 [349 → 305]
2	46.22	20-O-β-Glucopyranosyl-18-hydroxycamptothecin	527	nd
3	27.31	10-Hydroxycamptothecin	365	MS^2 [365 → 321]
4	nd	20-Hexanoyl-10-methoxycamptothecin	477	nd
5	nd	Mappicine	307	nd
6	27.84	20-Formylbenz-6,7-indolizino-1,2-quinoline-11-(13H)-one	313	nd
7	33.93	10-Methoxycamptothecin	379	MS^2 [379 → 335]
8	36.89	20-Deoxycamptothecin	333	MS^2 [333 → 289]
9	35.91	Vincosamide	499	MS^2 [499 → 337]
10	24.44	Strictosidinic acid	517	MS^3 [517 → 499 → 355]
11	21.05	Pumiloside	513	MS^2 [513 → 351]
12/13	40.01 or 39.98	3,4-Methylendioxy-3′-O-methyl-5′-hydroxyellagic acid or 3,3′,4-Tri-O-methylellagic acid	345	MS^3 [345 → 330 → 313]
14	46.27	3,3′,4-Tri-O-methylellagic acid-4′-O-D-glucopyranoside	507	nd
15	45.03	3,4-Methylenedioxy-3′-O-methyl-4-glucopyranosyl ellagic acid	491	MS^2 [494 → 419]
16	41.64	3,4-Methylenedioxy-3′,4′-O-dimethyl-5′-methoxyellagic acid	373	MS^2 [373 → 358]
17	21.03	3,4-Methylenedioxy-3′,4′-O-dimethyl-5,5′-dimethoxyellagic acid	403	nd
18	38.63	Quercetin	303	MS^2 [303 → 257]
19	26.99	Quercetin-3-O-D-galactopyranoside	465	MS^2 [465 → 303]
20	26.99	Quercetin-3-O-D-glucopyranoside	465	MS^2 [465 → 303]
21	28.35	Kaempferol-3-O-D-galactopyranoside	449	MS^2 [449 → 287]
22	39.98	Kaempferol-3-O-D-glucopyranoside	449	MS^2 [449 → 287]
23	24.92	Rutin	611	MS^3 [611 → 465 → 303]
24	42.11	Kaempferol	287	MS^2 [287 → 241]

nd: not detected.

Table 2
Occurrence of compounds **1**–**24** in extracts of leaves and leaf buds of *Camptotheca acuminata*.

	Compound	MW	Freeze-dried leaves 2004	Freeze-dried leaves 2003	Air-dried leaves 2003	Air-dried leaves 13/10/2004	Freeze-dried leaf buds 2004
1	Camptothecin	348	x	x	x	x	x
2	20-O-β-Glucopyranosyl-18-hydroxycamptothecin	526		x		x	x
3	10-Hydroxycamptothecin	364				x	
4	20-Hexanoyl-10-methoxycamptothecin	476					x
5	Mappicine	306					x
6	20-Formylbenz-6,7-indolizino-1,2-quinoline-11-(13H)-one	312					x
7	10-Methoxycamptothecin	378	x	x	x	x	x
8	20-Deoxycamptothecin	332	x	x			
9	Vincosamide	498		x	x	x	x
10	Strictosidinic acid	516	x	x	x	x	x
11	Pumiloside	512	x	x		x	
12/13	3,4-Methylendioxy-3′-O-methyl-5′-hydroxyellagic acid or 3,3′,4-Tri-O-methylellagic acid	344			x	x	
14	3,3′,4-Tri-O-methylellagic acid-4′-O-D-glucopyranoside	506	x				
15	3,4-Methylenedioxy-3′-O-methyl-4-glucopyranosyl ellagic acid	490	x			x	
16	3,4-Methylenedioxy-3′,4**′**-O-dimethyl-5′-methoxyellagic acid	372				x	
17	3,4-Methylenedioxy-3′,4′-O-dimethyl-5,5′-dimethoxyellagic acid	402		x	x		x
18	Quercetin	302	x			x	
19	Quercetin-3-O-D-galactopyranoside	464	x	x	x	x	x
20	Quercetin-3-O-D-glucopyranoside	464	x	x	x	x	x
21	Kaempferol-3-O-D-galactopyranoside	448	x	x	x	x	x
22	Kaempferol-3-O-D-glucopyranoside	448	x	x	x	x	x
23	Rutin	610					x
24	Kaempferol	286				x	

x: compound present.
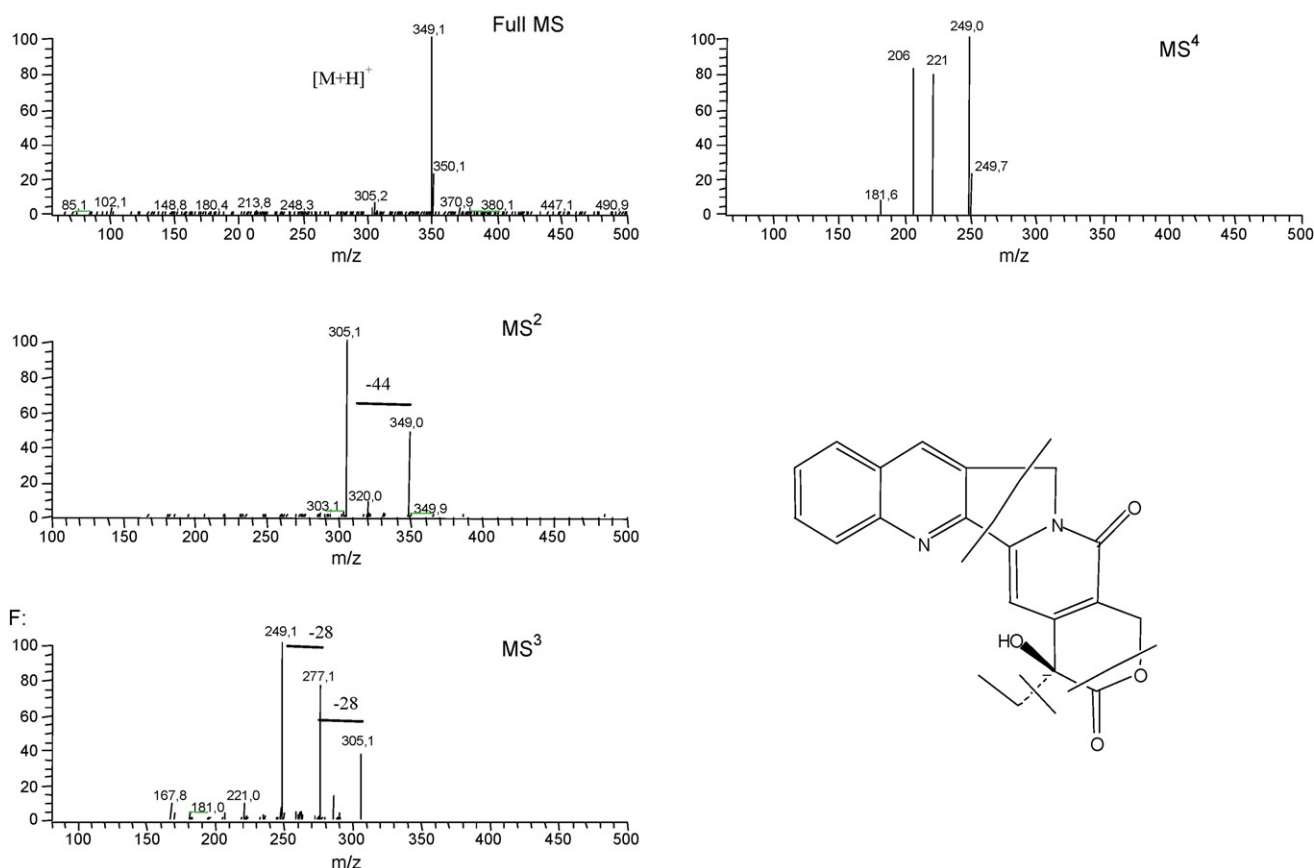
Fig. 4. ESI-IT-MS^n spectra of camptothecin.

corresponding to the protonated molecular ions of alkaloids, ellagic acid derivatives and flavonoids. Individual components were identified by comparison of their m/z values in the TIC profile with those of the selected compounds described in literature. RICs were generated for specific compounds expected to be present in the sample, and the following constituents were identified: **1** (t_R 31.04, m/z 349), **2** (t_R 46.22, m/z 527), **3** (t_R 27.31, m/z 365), **7** (t_R 33.93, m/z 379), **9** (t_R 35.91, m/z 499), **10** (t_R 24.44, m/z 517), **11** (t_R 21.05, m/z 513), **12** (t_R 40.01, m/z 345), **13** (t_R 39.98, m/z 345.5), **15** (t_R 45.03, m/z 491),

16 (t_R 41.64, m/z 373), **18** (t_R 38.63, m/z 303), **19** or **20** (t_R 26.99, m/z 465), **21** (t_R 28.35, m/z 449), **22** (t_R 39.98, m/z 449), **23** (t_R 24.92, m/z 611), and **24** (t_R 42.11, m/z 287). Additional HPLC–ESI-MS/MS experiments were carried out using the dependent scanning mode in which the MS software, based on parameters already fixed by the operator, selects ions of a certain intensity and submits those ions to fragmentation experiments using the parameters previously chosen. On this basis, the identities of compounds **1–24** were confirmed from the MS^2 and MS^3 data (Table 1).

Fig. 5. ESI-QqQ-MS/MS spectrum of camptothecin.

Fig. 6. Proposed fragmentation patterns for camptothecin induced by ESI-IT-MS/MS and ESI-QqQ-MS/MS.

This form of qualitative analysis provides a valuable fingerprint of the major metabolites present in leaf samples of *C. acuminate*. Detailed HPLC analyses revealed that the presence of selected components amongst compounds **1–24** was specific for each sample of *C. acuminate*, as shown 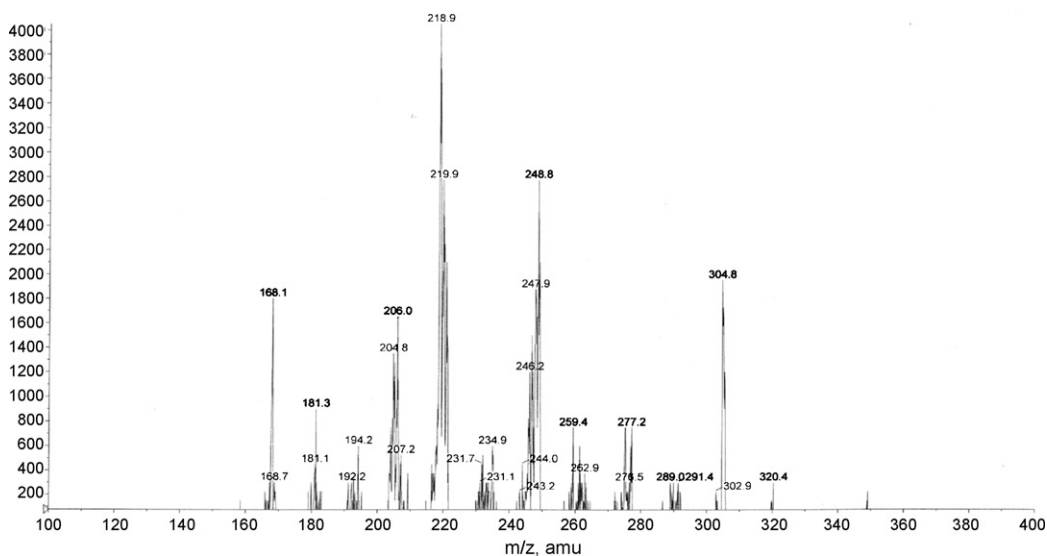in Table 2. Such variation could be due to different periods of plant development, different climatic conditions during cultivation, different times of harvesting, or different drying modes.

3.2. ESI-MS and ESI-MS/MS of CPT

No data concerning the MS fragmentation of CPT under ESI-IT-MS/MS (tandem mass spectrometry in time) or ESI-QqQ-MS/MS (tandem mass spectrometry in space) conditions are currently available in the literature. In the present study, a solution of CPT in water:methanol (1:1; acidified with 1% acetic acid) was infused directly into the ionisation source with the aid of a syringe pump in order to assess the behaviour of the alkaloid under the ESI-IT-

MS and ESI-IT-(MS)^n conditions as described above. Fig. 4 shows a series of sequential fragmentations of CPT obtained by ESI-(MS)^n in the positive ion mode. The predominant ion in the ESI-MS spectrum (not reported) was the protonated molecular ion ([M+H]$^+$, m/z 349) produced by the protonation of CPT. In the MS^2 spectrum, the base peak was a fragment ion ([M+H-44]$^+$, m/z 305) generated by the loss of 44 amu presumably corresponding to a neutral molecule of carbon dioxide. Other fragments appeared in the MS^3 and MS^4 spectra at m/z 277, 249 and 221 (Fig. 4). The first two of these presumably derive from the subsequent loss of neutral molecules such as ethylene and carbon monoxide, respectively, while the third could result from fragmentation of the molecular skeleton leading to a rearrangement of the molecule itself. These results may be compared with the fragmentation pattern obtained for CPT using ESI-QqQ-MS and ESI-QqQ-MS/MS (Fig. 5). In this case the ESI-MS base peak was again the protonated molecular ion ([M+H]$^+$, m/z 349) obtained by protonation of CPT, while the ESI-MS/MS spectrum displayed fragment ions at m/z 305 [H+M-44]$^+$ and 249 [H+M-44-CO–C$_2$H$_5$]$^+$
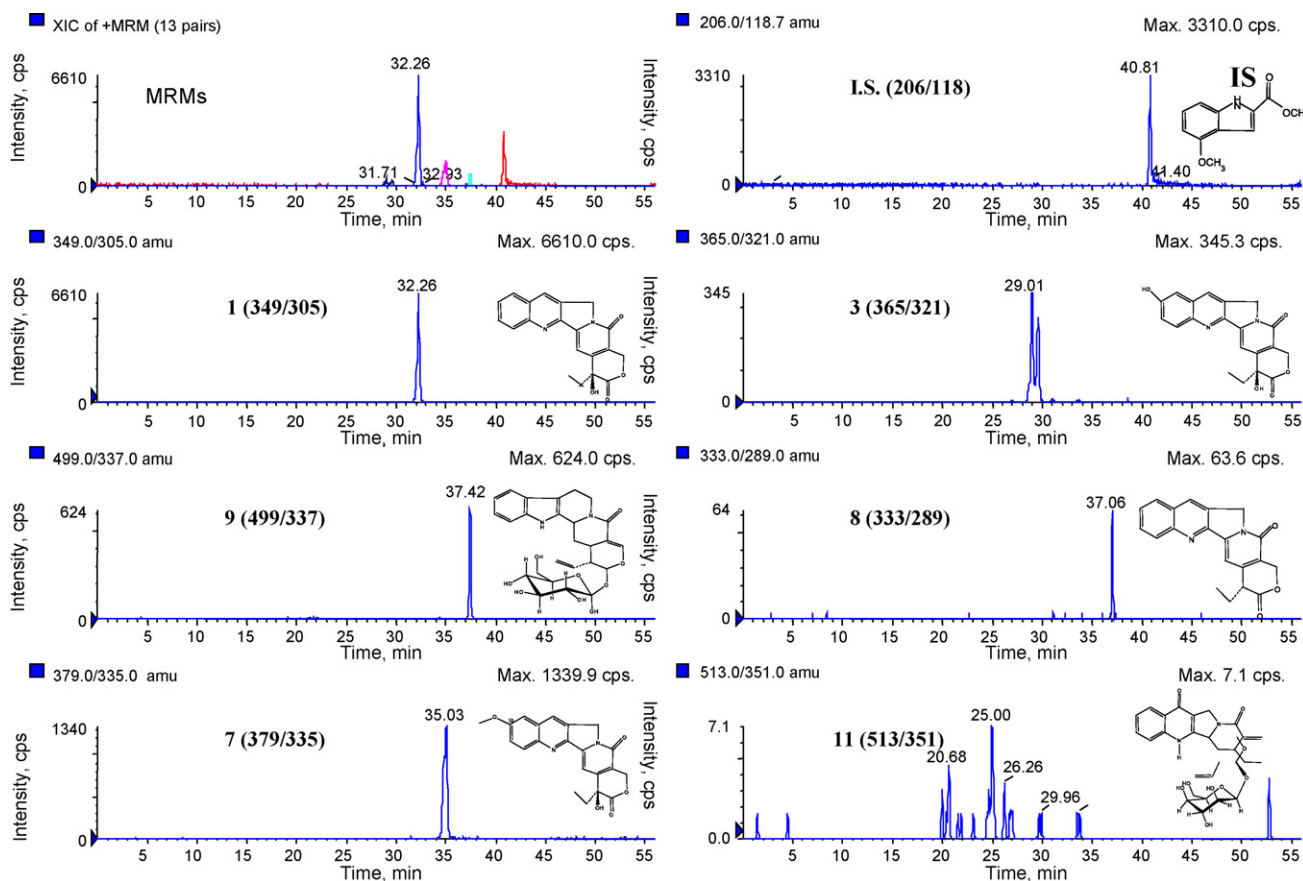
Fig. 7. Fragment transitions employed to monitor the alkaloids in samples of *C. acuminata* by MRM and for their quantitative determination by HPLC–ESI-QqQ-MS/MS.

together with a very intense base peak at *m/z* 219. This latter fragment, which was not observed in the ESI-IT-MS/MS analysis, is supposed to be originated by rearrangement of the molecule subsequent to the loss of one molecule of formaldehyde from the ion at *m/z* 249. The suggested fragmentation schemes are presented in Fig. 6.

3.3. Quantitative HPLC–ESI-QqQ-MS/MS analyses of C. acuminata extracts

It is recognised that tandem mass spectrometry in space provides a better accuracy in quantitative analyses than tandem mass spectrometry in time [21]. On this basis, a mass spectrometer equipped with a triple quadrupole analyser was employed in the elaboration of a quantitative assay for the alkaloids in *C. acuminata* extracts. It was observed that the base peaks in the HPLC–ESI-IT-MS/MS-generated MS^2 spectra of the alkaloids generally comprised the fragment obtained by loss of carbon dioxide from the protonated molecular ion. On the basis that HPLC–ESI-QqQ-MS/MS-generated spectra would be similar, the specific fragmentation reaction $[M+H]^+ \rightarrow [M+H-CO_2]^+$ was selected in order to monitor the alkaloids by MRM. However, for compounds **9** and **11**, which were characterised by their glycosylated structures, the loss of a sugar unit was the predominant fragmentation, and these alkaloids were monitored by the transition from the specific protonated molecular ion $[M+H]^+$ to the corresponding aglycone ion $[A+H]^+$. Compounds **1**, **3**, **7–9** and **11** could be detected with high sensitivity in samples of *C. acuminata* using the reported MS conditions. Considering that these are the major alkaloids present in this species, further quantification focused on these compounds. The transitions employed to monitor these alkaloids are summarised in

Fig. 7. Under the conditions of HPLC–ESI-MS/MS, IS (internal standard, methyl 4-methoxy-2-indolecarboxylate) was characterised by MRM through the transition from precursor ion *m/z* 513.0 to product ion *m/z* 351.0. All of these compounds were determined on the basis of the calibration curve for the quantification of CPT. This curve was constructed by plotting the area ratio between the external and internal standard against the known concentration of CPT (Fig. 8), and was found to be linear in the range 5–60 μg/mL.

3.4. Validation of the method

The HPLC–MS/MS assay was validated according to the European Medicines Agency (EMEA) guidelines relating to the validation of analytical methods [22]. The method based on the characteristic fragmentation reactions of alkaloids and alkaloid glycosides was highly specific with no other peaks interfering at the retention times of the marker compounds (i.e. **1**, **3**, **7–9**, and **11**) in the MRM chromatograms. The intra-day accuracy and precision were calculated by analysing three samples of CPT at three different concentration levels, namely, 1, 5 and 10 μg/mL, on the same day. Inter-day estimates were performed over three consecutive days. The standard deviation was <5%. The calibration graph, obtained by plotting the area ratio between the external and internal standard against the known concentration of external standard (CPT), was linear in the range of 5–60 μg/mL. The limit of quantification (LOQ), defined as the lowest concentration of compound quantifiable with acceptable accuracy and precision, was determined by injection of a series of diluted standard solutions until a signal-to-noise ratio of 10 was attained. The LOQ values calculated for CPT were less than 15 ng/mL.
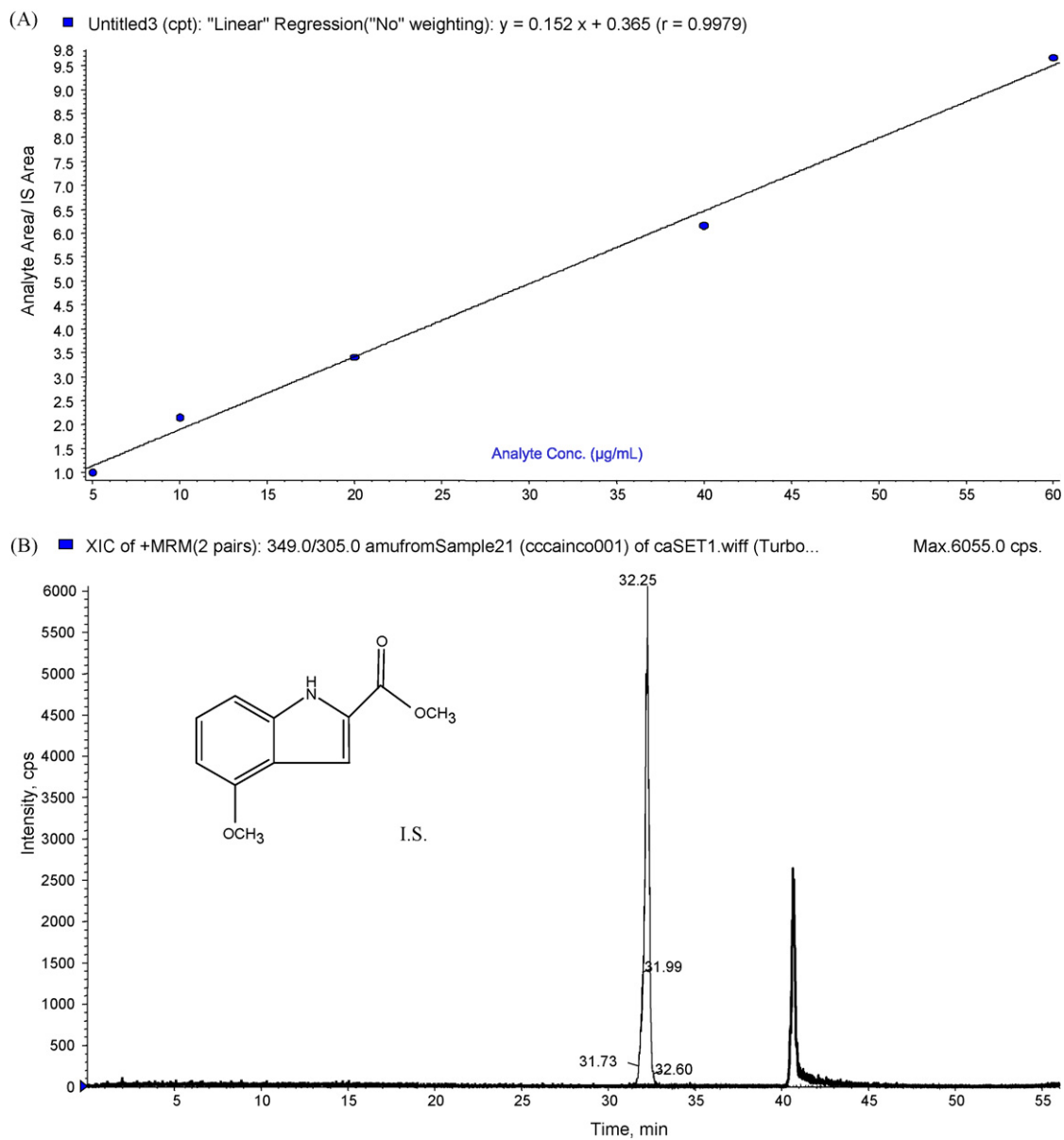
Fig. 8. (A) Calibration curve for the quantitative determination of camptothecin in extracts of *C. acuminata* by HPLC–ESI-QqQ-MS/MS. (B) MRM analysis of CPT and internal standard at concentrations of 20 and 10 μg/mL, respectively.

3.5. Alkaloids in C. acuminata extracts

Five aliquots of each extract of *C. acuminata* were analysed in order to determine the content of **1**, **3**, **7–9** and **11**, and the results are reported in Table 3. Freeze-dried leaves and leaf buds collected in summer 2004 contained higher levels of alkaloids than freeze-dried leaves collected in 2003 or air-dried leaves collected in 2004. The higher CPT content of leaf buds and young leaves

Table 3
Content of camptothecin and related alkaloids in extracts of leaves and leaf buds of *C. acuminata*.

	Freeze-dried leaf buds 2004 (mg/g plant)	Freeze-dried leaves 2004 (mg/g plant)	Freeze-dried leaves 2003 (mg/g plant)	Air-dried leaves 13/10/2004 (mg/g plant)
1	3.504 ± 0.123	3.105 ± 0.110	0.941 ± 0.089	1.094 ± 0.109
3	nd	nd	nd	1.122 ± 0.103
6	2.465 ± 0.093	nd	nd	nd
7	0.059 ± 0.055	1.592 ± 0.094	0.056 ± 0.075	0.895 ± 0.099
8	nd	0.196 ± 0.147	0.125 ± 0.100	nd
9	0.215 ± 0.101	1.384 ± 0.108	1.115 ± 0.087	0.85 ± 0.054
10	0.302 ± 0.074	0.302 ± 0.110	0.75 ± 0.014	0.932 ± 0.097
11	nd	0.512 ± 0.121	0.608 ± 0.045	0.221 ± 0.023
Total alkaloids	6.545 ± 0.089	7.091 ± 0.115	3.595 ± 0.069	5.114 ± 0.081

nd: not detected.

may reflect the requirement for a robust chemical defence system by these delicate tissues. Biosynthetic precursors of CPT were found to be present in large amounts in all of the extracts studied. The presence of compounds **3**, **7** and **8**, which probably arise from oxidation processes, in some of the studied extracts is particularly noteworthy. Another compound that was found to be present in remarkable quantities was 20-formylbenz-6,7-indolizino-1,2-quinoline-11-(13H)-one (6). This molecule is believed to be formed by the biosynthetic modification of CPT with a terminal aromatic ring that is more stable than the lactone present in the original structure.

4. Conclusions

Leaves of *C. acuminata* analysed at different growth stages showed considerable variation in their accumulation of CPT, alkaloid derivatives and phenolic compounds, with young leaves showing the major alkaloids content. Such altered profiles may reflect different accumulation patterns in the plants. Various alkaloids related to CPT were present in the studied extracts in large amounts, some of them in quantities comparable with that of CPT itself. While the medicinal value of these alkaloids has yet to be established, the different compositions determined in this study could be tested for synergism as suggested by Zhang et al. [23]. Conversely, it would be interesting to investigate the anti-tumour potential of these natural products and to determine their possible use as precursors of semi-synthetic derivatives of CPT.

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