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Determination of quinolizidine alkaloids in different *Lupinus* species by NACE using UV and MS detection

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ABSTRACT

Lupin seeds are important for animal and human nutrition. However, they may contain toxic quinolizidine alkaloids (QA). Analytical methods for a reliable alkaloid determination are therefore of importance. Here the presented study reports on the first CE method for the analysis of QA in *Lupinus* species. A buffer system consisting of 100 mM ammonium formate in methanol, acetonitrile, and small amounts of water and acetic acid enabled the baseline separation of sparteine, lupanine, angustifoline and 13α-hydroxylupanine in less than 10 min. Applied voltage, temperature and detection wavelength were 25 kV, 30 °C and 210 nm, respectively. Additional compounds were identified in CE–MS experiments, in which all alkaloids could be assigned in positive ESI mode at corresponding $[M+H]^+$ values. The CE method was validated for linearity, sensitivity, accuracy and precision, and then used to assess the seeds of seven different *Lupinus* species for their alkaloid content. Lupanine was present in all of them within a range from 0.02% (*L. densiflorus*, *L. microcarpus*) to 1.47% (*L. albus*). The highest percentage of an individual alkaloid was found in *L. polyphyllus* (3.28% of angustifoline), the content of total alkaloids ranged from 0.43% (*L. microcarpus*) to 5.13% in *L. polyphyllus*. The quantitative results were in good agreement with literature data.

1. Introduction

Just like soybeans lupin seeds have a high protein content of 30–40% in dry matter, thus they are considered as an interesting alternative food source to replace for example genetically modified soy [1]. Lupin and soy both belong to the Fabaceae, and out of approx. 400 *Lupinus* species known four have been domesticated and are of commercial interest: *L. albus* (white lupin), *L. luteus* (yellow lupin), *L. angustifolius* (narrow leaf lupin) and *L. mutabilis* (Andean lupin) [2]. Lupin flour or products might be of health concern because of two reasons. Firstly, the seed proteins may show crossallergy with peanut proteins (therefore both are considered food allergens, although lupin proteins are weaker allergens than peanut or soybeans [3]), and secondly, lupin seeds of wild varieties can contain up to 6% of bitter tasting quinolizidine alkaloids (QA). The latter should be no major health risk anymore, because nowadays only "sweet lupins" are cultivated as crops. These are special plant varieties that contain less than 0.02% alkaloids making respective products safe for human and animal consumption [2].

Lupins contain quinolizidine alkaloids like sparteine, lupanine or angustifoline (see Fig. 1) as defence chemicals against insects and other herbivores. Intoxications with these compounds in mammals cause convulsions, shaking and trembling, and death from respiratory and cardial arrest. QA modulate both nicotinic and muscarinic acetylcholine receptors, some inhibit sodium channels and possibly lead to neuronal degenerations [4–6]. The well known "crooked calf disease" is mainly caused by other toxic quinolizidine alkaloids, anagyrine or ammodendrine, which do not occur in the domesticated *Lupinus* varieties. It is well documented that alkaloid composition and alkaloid levels vary under different environmental conditions [4,7]. Thus, it is definitely of interest to control the amount of alkaloids in lupin plant material and products. Most commonly GC or GC–MS are utilized for this purpose [8–12], compared to a rather small number of respective HPLC reports [12,13]. The successful use of more unspecific and less sensitive approaches like photometry [14] and TLC [15] has been described as well.

What never has been attempted is the analysis of *Lupinus* alkaloids by CE (there is one publication describing the separation of isoflavones in *L. albus* and *L. angustifolius* by CE only [16]). This is somehow surprising because this technique is especially suitable for chargeable compounds like proteins and alkaloids. In addition to high separation efficiency and economic use it is of great methodological versatility, such as non-aqueous capillary electrophoresis (NACE) or the possibility of hyphenation to a mass spectrometer

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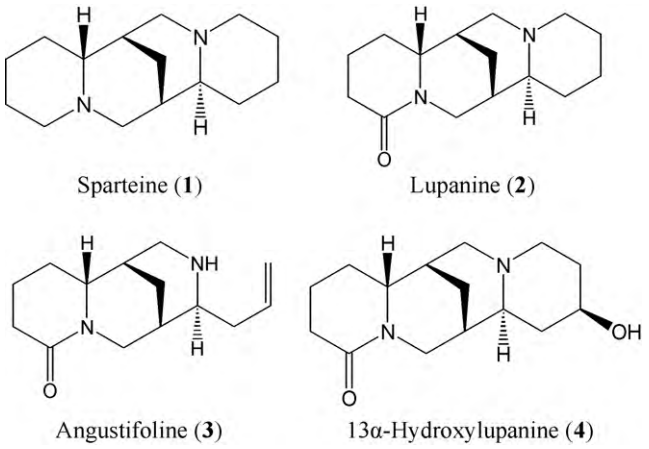

Fig. 1. Chemical structures of the four quinolizidine alkaloid standards.

(CE–MS) [17]. These two approaches were employed in this study to separate, detect and quantify quinolizidine alkaloids in different *Lupinus* species.

2. Experimental

2.1. Materials

Authenticated plant material (seeds of seven different *Lupinus* species; see Table 2 for further details) assayed in this study and three reference compounds (lupanine, angustifoline and 13α-hydroxylupanine) were provided by one of the authors (M. Wink). Sparteine was bought from Extrasynthese (Genay, France). Voucher specimens of the plant material are deposited at the herbarium of the Institute of Pharmacy, Pharmacognosy, at the University of Innsbruck.

All chemicals (ammonium formate, acetic acid, 0.5N hydrochloric acid, 1N sodium hydroxide solution) and solvents (methanol, acetonitrile, dichloromethane) used were of analytical grade and purchased from Merck (Darmstadt, Germany); ultrapure water was produced by a Sartorius Arium® 611 UV water purification system (Göttingen, Germany).

2.2. Sample preparation

The samples (5 g of dried seeds) were ground to a fine powder in a commercial coffee mill, and then 0.25 g of the plant material was homogenized two times with 8 ml 0.5N HCl in a sonicator for 30 min. The sample was centrifuged (3000 rpm, 10 min), the supernatants combined, and the pH of the solution adjusted to 10.0 with 1N NaOH. Afterwards, the aqueous solution was partitioned with 20 ml dichloromethane, the organic layer removed (sometimes centrifugation was required to separate both phases), and the same procedure repeated three more times. The organic layers were combined, the solvent evaporated under vacuum, and the residue dissolved in 10 ml methanol. Prior to injection all samples were filtered through a 0.45 μm nylon membrane filter (Minisart, Sartorius, Göttingen, Germany); each sample solution was analysed in triplicate. The sample solutions were stable for at least for three weeks if stored at 4 °C (confirmed by re-assaying).

2.3. Analytical method

Analytical experiments were performed on a 3D-CE system from Agilent (Waldbronn, Germany), equipped with autosampler, diode array detector (DAD) and temperature controlled column compartment. Capillaries (fused silica, 50 μm I.D., 52 (CE)/90 (CE–MS) cm effective length) were from Polymicro Technologies (Phoenix, AZ, USA).

The following buffer provided for the optimum CE separation of *Lupinus* alkaloids: a 100 mM solution of ammonium formate in a

Table 1
Performance characteristics of the developed CE assay.

Parameter	Compound			
	1	2	3	4
Regression equation	$y = 0.020x$	$y = 0.242x$	$y = 0.039x$	$y = 0.299x$
σ_{rel} of slope	4.32	3.67	5.70	2.89
Correlation coefficient	0.998	0.999	0.999	0.999
Plate number (n)	191,000	190,000	224,000	155,000
Range (μg/ml)	55–1500	17–1400	18–1460	17–1400
LOD (μg/ml)	30.5	7.9	16.1	6.5
LOQ (μg/ml)	87.0	18.2	61.4	12.5
Accuracy (high spike)[a]	–	99.3%	–	–
Accuracy (medium spike)[a]	–	98.8%	–	–
Accuracy (low spike)[a]	–	97.9%	–	–
Precision (intra-day)[b]	6.42	4.67	3.75	4.23
Precision (inter-day)[c]	4.38	4.23	3.95	1.76

[a] Expressed as recovery rates, only determined for lupanine.
[b] Maximum deviation within one day based on peak area in percent ($n = 5$).
[c] Deviation within three days based on peak area in percent.

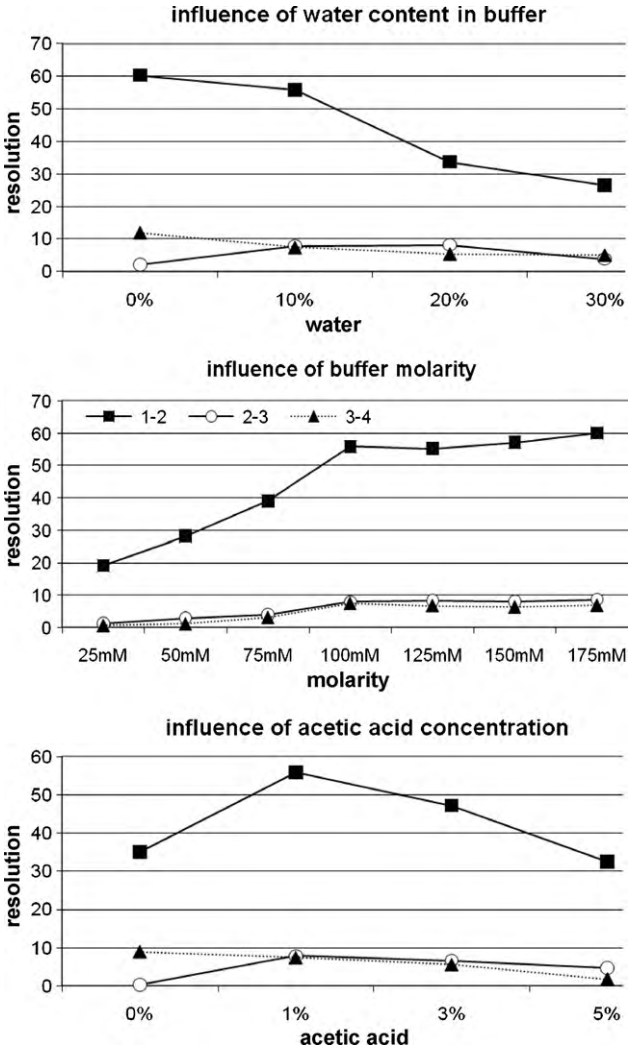

Fig. 2. Influence of water content, buffer molarity and concentration of acetic acid on the resolution of adjacent peaks.

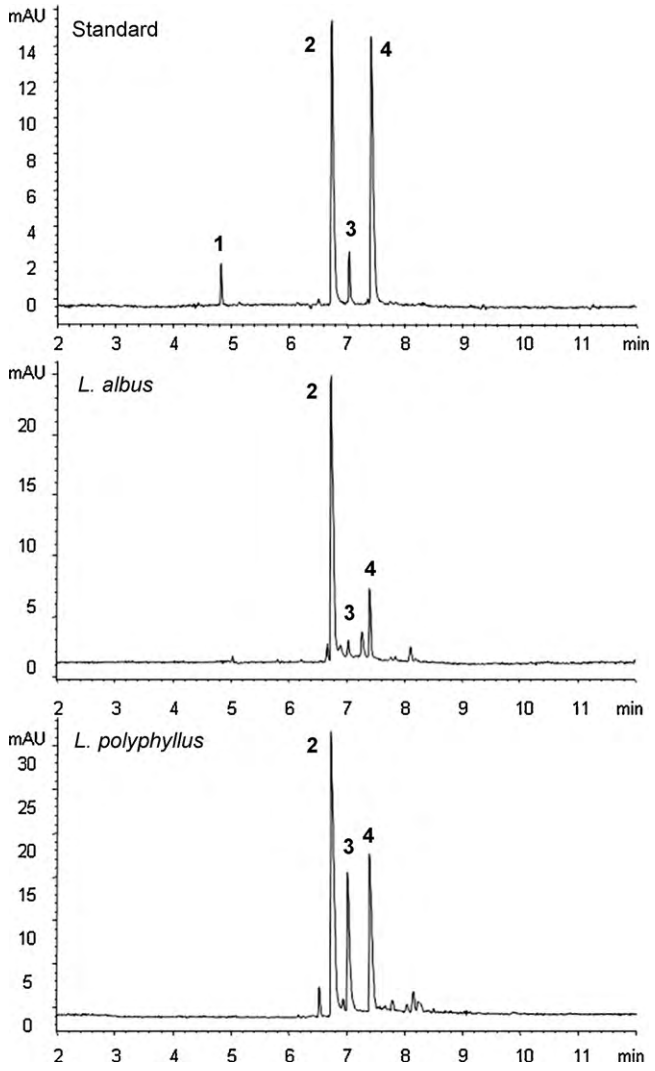

Fig. 3. Analysis of a standard mixture and the extracts of two *Lupinus* species under optimized CE conditions (fused-silica capillary 60 cm × 50 μm I.D.; effective length 52 cm; 100 mM ammonium acetate in MeOH/ACN/water (70/20/10) with 1% HOAc; hydrodynamic injection at 50 mbar for 2 s; separation voltage: 25 kV; temperature: 30 °C; detection wavelength: 210 nm); peak assignment according to Fig. 1.

mixture of methanol, acetonitrile, and water (70/20/10), which also contained 1% acetic acid. Applied voltage and temperature were 25 kV and 30 °C; samples were injected in hydrodynamic mode (50 mbar for 2 s). Over a runtime of 15 min the analysis was monitored at 210 nm, and between runs the capillary was flushed for 2 min with 0.1N NaOH, followed by buffer (2 min). New capillaries were rinsed with 0.1N NaOH, 0.01N NaOH and water (30 min each) prior to initial use. Before use all buffers and washing solutions were membrane filtered.

For CE–MS experiments the outlet end of the capillary was connected to an Esquire 3000 plus iontrap MS from Bruker (Bremen, Germany) via specific interface from Agilent. Except the need of a sheath liquid (50% aqueous methanol containing 0.1% acetic acid), which was introduced to the MS-sprayer at a flow rate of 0.2 ml/h by syringe pump (74900 series, Cole-Parmer, Vernon Hills, IL, USA), the same analytical conditions as mentioned above were applied. All alkaloids were assignable in positive ESI mode, with probe temperature, spray voltage, sheath gas and dry gas set to 250 °C, 4.5 kV, 4 psi (nitrogen), and 4 l/min (nitrogen), respectively. For CE–MS analyses the capillary was only flushed with buffer during individual runs, in case of shifting migration times the capillary was disconnected from the MS interface and flushed with 0.1N NaOH for 5 min.

2.4. Calibration

A stock solution of QA was prepared by dissolving each of the four standards in methanol (1.5 mg/ml). This solution was 1:2 serially diluted with methanol in order to prepare further calibration levels, so that the linearity of the detector signal was confirmed from 1400 to 55 μg/ml (see Table 1 for detailed data). The standard solutions were stable for at least three weeks if stored at 4 °C (confirmed by re-assaying).

2.5. Method validation

For the determination of limit of detection (LOD) and limit of quantitation (LOQ) standard solutions were diluted to appropriate concentrations and after analysis the electropherograms were evaluated visually; LOD and LOQ are defined as 3- and 10-times baseline noise.

Repeatability was deduced from relative standard deviations for multiple injections well below 5.0%. Precision was assured by assaying one sample (*L. albus*) in quintuplicate on day 1. The same procedure was repeated on two more days, and the variation within one day (intra-day precision) and within three days (inter-day precision) was determined based on peak area. The methods accuracy was investigated by spiking plant material (*L. albus*) with known amounts of lupanine at different concentration levels (high, medium and low spike); other alkaloids were not used because only insufficient amounts were available. After extraction under optimized conditions and CE-analysis the obtained quantitative results were compared with the theoretically present ones (expressed as recovery rate). All validation results are combined in Table 1.

3. Results and discussion

The analysis of quinolizidine alkaloids like matrine or oxymatrine (both can be found in *Sophora* species) by CE has been described using aqueous [18] and non-aqueous buffer systems [19]. In this study we evaluated both approaches, but initial screening experiments already indicated that non-aqueous buffer systems are more suitable for the current separation problem. Aqueous electrolyte solutions were not compatible for MS analysis (e.g. phosphate buffers), the achieved separations/peak symmetries were not satisfactory or an unstable electric current was observed. The latter was possibly caused by solubility problems of the alkaloids. Non-aqueous systems such as ammonium formate or ammonium acetate in methanol were apparently much better suited, but only after a careful evaluation of all parameters an acceptable separation of the standard compounds (see Fig. 1 for structures) was achieved. Formate buffers were preferred because they guaranteed a more stable baseline and decreased migration times.

Influences of the most relevant separation parameters are illustrated in Fig. 2, they are water content, buffer molarity and acetic acid concentration. Without adding a small percentage of water (10%) and acetic acid (1%) to the electrolyte solution lupanine and angustifoline could not be resolved. This can be explained by a different electrophoretic mobility of the alkaloids in charged versus neutral state. On the other hand, if the content of water and/or acetic acid is too high a general decline in resolution was observed. Increasing water resulted in impaired peak shape, increasing acid caused reduced migration times, both having a negative effect on the resolution of adjacent peaks. Using a methanol/acetonitrile mixture instead of pure methanol the same effect on migration times was observed, yet having no drawback on resolution; 20% of

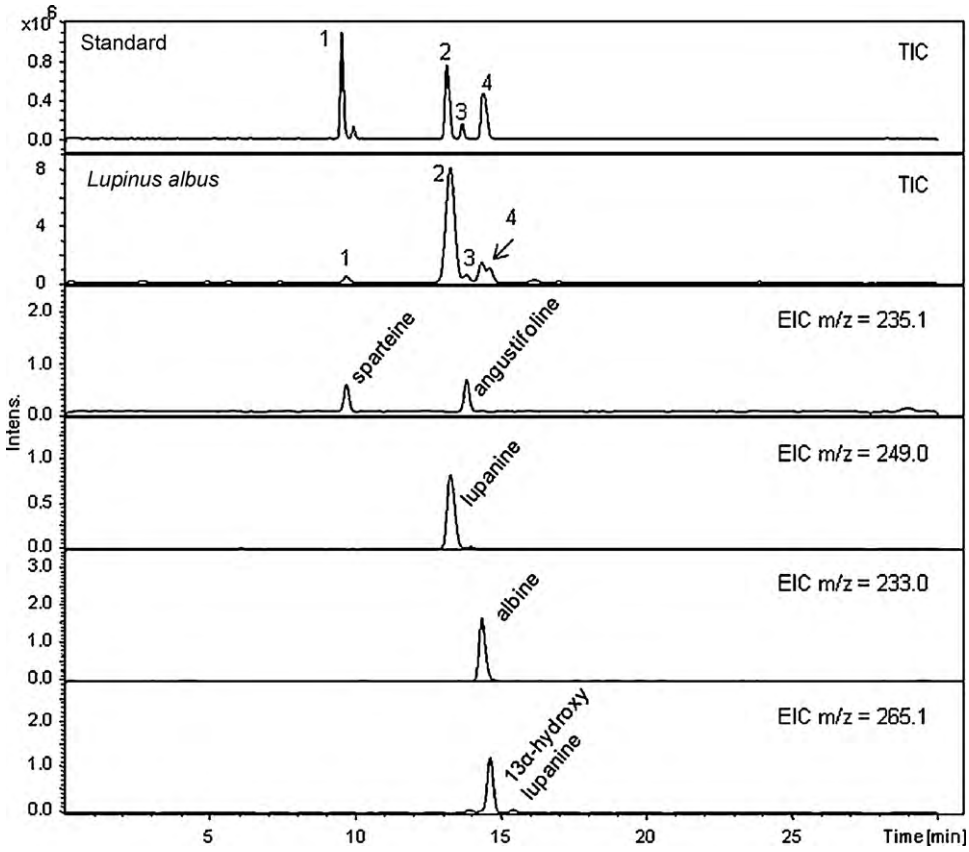

Fig. 4. CE–MS analysis of a standard solution (TIC: total ion current) and a *Lupinus albus* extract (TIC and EIC: extracted ion current). Analytical conditions as Fig. 2, except capillary length 90 cm. MS-conditions: ESI positive; sheath gas: 4 psi; dry gas: 4 l/min; probe temperature: 250 °C; spray voltage: 4.5 kV; sheath liquid: methanol/water = 1/1, with 0.1% acetic acid; sheath liquid flow rate: 0.20 ml/h.

acetonitrile showed the best results in this respect. The third most crucial factor was buffer molarity. It is well known that the electroosmotic flow decreases with an increase of ionic strength. With 25–75 mM ammonium formate solutions lupanine, angustifoline and 13α-hydroxylupanine simply migrate too fast for a baseline separation, with 100 mM buffers and higher a baseline separation could be achieved. Even though the resolution was slightly better at higher molarities a 100 mM ammonium formate buffer was finally selected due to a lower current (see CE–MS experiments described below). Temperature and applied voltage had only an effect on migration times but not on peak resolution.

Separation of a standard mixture and the extracts of two *Lupinus* species (*L. albus* and *L. polyphyllus*) under optimized CE conditions are shown in Fig. 3. Within less than 10 min sparteine, lupanine, angustifoline and 13α-hydroxylupanine could be well separated with a minimum resolution of 7.5, and the assignment of major signals in the extracts was easily possible based on migration times and UV-spectra. For identification of other constituents CE–MS experiments proved to be very helpful. As the selected buffer only consisted of volatile constituents it readily could be used for MS as well, the choice of a 100 mM buffer showed to be optimal as the resulting current of 35 μA was compatible with the respective upper limit of the MS (40 μA). The only modifications were an increased capillary length (caused by the gap between CE and MS) and the need for a sheath liquid. In CE–MS experiments the capillary outlet is directly coupled to the MS. As the minute CE-efflux of liquid would not provide for a stable current it is required to add a separate liquid, in this case a 1:1 mixture of water and methanol containing 0.1% acetic acid, to the ESI source. With this setup stable and reproducible analyses were possible, with all alkaloids being detectable in positive ESI mode at m/z values corresponding to $[M+H]^+$ (Fig. 4). Peak purity was assured by evaluating UV- and MS-spectra, and no indications for co-eluting compounds were found.

Method optimization was followed by the validation of the CE assay, for this purpose the methods linearity, sensitivity, accuracy and precision were determined. The results presented in Table 1 indicate a wide linear range from 50 to 55 μg/ml, combined with excellent correlation coefficients ($R^2 \geq 0.998$) and LOD/LOQ values typical for CE. Compared to HPLC the latter is usually less sensitive, a fact that easily can be explained by the extremely small sample volumes that are injected (only a few nl). The assays accuracy was determined in recovery experiments at three concentration levels, but as only small amounts of standards were available, only using lupanine. The recovery rates were close to 100% (97.9–99.3%), indicating not only accuracy of the assay but also integrity of the sample preparation procedure. Last but not least, the methods precision was determined based on repeated analyses of one sample over several days. With a maximum relative standard deviation of 6.4% this parameter can be considered valid as well.

At last seven *Lupinus* species, all of them non-cultivated ones, were evaluated for their alkaloid composition and content. In order to do that a suitable extraction procedure had to be selected, assuring a selective but also exhaustive extraction of the compounds of interest. Directly extracting the plant material with methanol did not show reproducible analyses, because of the high fatty oil content in the seeds migration times shifted from run to run and the observed peak symmetries were unacceptable. Thus, a “classical” alkaloid extraction was used. The compounds were first leached from the sample by an aqueous acidic solution, which was then

Table 2
Quinolizidine alkaloids and total of alkaloids (both in mg/g plant material) in diverse *Lupinus* species; relative standard deviation in percent in parentheses ($n = 3$).

Species	Compound					
	1	2	3	4	Others[a]	Σ of alkaloids
L. albus	–	14.70 (0.46)	5.95 (0.20)	2.05 (1.59)	Albine: 0.81 (0.49)	23.51
L. arboreus	22.81 (1.26)	1.73 (0.41)	–	–		24.54
L. densiflorus	–	0.25 (1.39)	0.10 (2.55)	0.23 (2.31)	Cytisine: 1.11 (1.56) Anagyrine: 0.24 (0.08) *N*-methylcytisine: 5.07 (3.93)	7.00
L. exaltatus	–	8.90 (0.56)	12.47 (0.10)	0.28 (1.23)		21.65
L. hartwegii	1.78 (0.10)	7.92 (2.91)	–	0.39 (0.33)		10.09
L. microcarpus	0.99 (0.37)	0.23 (1.84)	–	–	Anagyrine: 0.81 (0.96) *N*-methylcytisine: 2.23 (1.90)	4.26
L. polyphyllus	–	10.83 (0.62)	32.80 (0.93)	7.67 (0.78)		51.30

[a] Tentatively identified based on MS data, quantified based on calibration curve of lupanine.

adjusted to a basic pH, so that the alkaloids were extractable with an organic solvent. This procedure allowed the exhaustive extraction of alkaloids as indicated by recovery rates close to 100%. The quantitative results obtained are summarized in Table 2. Each of the extracts was assessed three times, and the resulting relative standard deviations were all below 3%. All of the samples contained lupanine, in amounts ranging from 0.02% (*L. densiflorus*, *L. microcarpus*) to 1.47% (*L. albus*). Except *L. arboreus*, *L. hartwegii* and *L. microcarpus* all species also contained angustifoline (0.01–3.28%) and 13α-hydroxylupanine (0.02–0.77%); sparteine was confirmed in three species only. As mentioned above the sensitivity of CE assays is generally lower than HPLC or GC. Yet, the results obtained indicate that the developed assay allows the determination of individual alkaloids lower than the tolerated total alkaloid content in food (0.02%; [2]). In addition to these four compounds, which were available as standards, other alkaloids were tentatively assigned based on their molecular mass [8]. For example in *L. densiflorus* the presence of three other quinolizidine alkaloids was confirmed, namely cytisine, anagyrine and *N*-methylcytisine. In order to compare the total alkaloid content of the different species their amount was calculated based on the calibration curve of lupanine. This showed that the seeds of *L. microcarpus* contain the overall lowest amount of alkaloids (0.43%), whereas *L. polyphyllus* is rather rich in these toxic natural products (5.13%).

4. Conclusions

The here presented CE/CE–MS assay is shown to be well suited for the qualitative and quantitative analysis of quinolizidine alkaloids in lupin seeds. It combines CE typical benefits like high separation speed (analysis time of 8 min compared to 30 min by GC [11] or 25 min by HPLC [13]), excellent efficiency ($n \geq 150{,}000$) and economic performance (minute sample and buffer consumption) with broad applicability. The latter refers to the successful analysis of different *Lupinus* species as well as the use of an MS as additional detector, which permitted the identification of all major alkaloids. The assay fulfils all validation criteria and the quantitative results obtained are in good agreement to published data [8,11]. For example, the relative alkaloid composition in *L. densiflorus* has been described with lupanine (<1%), cytisine (11%), anagyrine (1%) and *N*-methylcytisine (76%) [8]. Our results showed comparable values of 2%, 15%, 3% and 72%, which are acceptable considering that a different sample batch was analyzed and the variability of biological material in general. Quantitative results showed a good degree of consensus as well. De Cortes et al. reported a total alkaloid content in *L. albus* of 2.36 g/100 g [20], we nearly found the same value (2.35%). All this clearly indicates that CE, for this application, can be considered an equal if not better analytical approach compared to established techniques like GC or HPLC.

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