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# UPLC TOF MS for sensitive quantification of naturally occurring pyrrolizidine alkaloids in *Petasites hybridus* extract (Ze 339)

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ABSTRACT

Due to increasing regulatory awareness of their hepatotoxic, genotoxic and possibly carcinogenic potential, pyrrolizidine alkaloid (PA) content has to be thoroughly monitored in herbal medicinal preparations. Recently, new very low PA regulatory threshold concentrations have been requested by the authorities. Therefore, a highly sensitive and reproducible UPLC TOF MS method for the quantification of the PAs senkirkine, senecionine, seneciphylline, senecionine-*N*-oxide and seneciphylline-*N*-oxide in a $CO_2$-extract of *Petasites hybridus* leaves (Ze 339) has been developed.

The limit of quantification (LOQ) was 2 ppb for all PAs. Recovery at the LOQ was between 88.9 and 141.9%, the repeatability precision between 3.5 and 13.6%. Linearity of the five PAs showed correlation coefficients between 0.9995 and 0.9998 and coefficients of variation between 7.44 and 8.56%. A working range between 2 ppb and 200 ppb could be fixed. In the tested batches of the *P. hybridus* extract Ze 339, the absence of PAs could be demonstrated. In conclusion, this assay allows to determine trace PA concentrations in *P. hybridus* extract Ze 339, making it suitable for analytical PA monitoring in accordance with regulatory requirements.



## 1. Introduction

Since, the discovery of 1,2-unsaturated pyrrolizidine alkaloids (PA) in medicinal plants and herbal medicines in the 1970s and the recognition of their toxicity to humans, such as veno-occlusive disease, genotoxicity and possibly cancer, there have been a huge number of publications for the quantitative analysis of these substances. At the beginning of PA analysis, gas chromatographic (GC) methods with flame ionization (FID) or nitrogen–phosphorus (NPD) detection as well as high pressure liquid chromatography (HPLC) methods with ultraviolet light (UV) detection had been used, preferentially. All of these methods, which are still used in PA analysis, are, however, confounded with some serious disadvantages. Thus, for example, the PA-*N*-oxides are not detected directly by gas chromatography, which necessitates their previous reduction to the corresponding free bases. HPLC-UV methods have the disadvantage that a low and non-specific wavelength (220 nm) is required to detect the unsaturated PAs and therefore, the choice of solvents is limited. Both types of chromatography, combined with conventional detectors cannot longer withstand the enormous increase in requirements for a high specificity and sensitivity in PA analysis. For these reasons, LC–MS-methods came more and more into focus, since they allow reliably identification of the analytes with a substantially improved sensitivity (for an overview of the different methodological approaches see [1]).

Regulatory authorities demand the reliable quantification of PAs in a very low concentration range (trace analysis) for herbal food and pharmaceutical preparations: In 2011, the European Food Safety Authority (EFSA) proposed a limit for the human daily intake of 1,2-unsaturated PAs of 70 μg/kg b.w. by food products [2]. The Australia New Zealand Food Authority (ANZFA) defined in 2001 a provisional tolerable daily intake for PAs in humans as 1 μg/kg b.w. [3]. In 2014, the British Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (COT) defined a safe dose of PAs of 0.007 μg/kg b.w./day [4]. For genotoxic impurities, a threshold value for lifelong oral intake of 1.5 μg/day was defined by the International Conference on Harmonization (ICH M7) [5]. Recently, the Committee on Herbal Medicinal Products (HMPC) of the European Medicines Agency (EMA) required a limit of 0.35 μg per day for short-term (≤14 days) oral administration from all sources (herbal medicinal preparations and food). Beyond this treatment period, no PAs are allowed [6]. Therefore, for a longer treatment with herbal preparations, the demonstration of absence of PAs is mandatory. However, this requires a highly sensitive and

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specific analytical method for the detection of PAs in the herbal extract.

Since, there are approximately 600 different PAs known, which naturally occur in different plants [2], an appropriate selection of PA reference standards is needed. In the case of *Petasites hybridus* (butterbur), the selection of relevant (naturally occurring) PA species was guided by previous reports of PA occurrence in this plant [7–9].

The determination of PAs in plant material or herbal extracts, especially at higher concentrations is considerably influenced by matrix effects. Among these, interferences of the PA-peaks with matrix substances with approximately the same mass often lead to false positive results, especially when single-stage MS was used. Matrix effects may be resolved first by the reduction of the concentration of test solutions, while using modern sensitive mass spectrometers [1], and secondly, by enhancing concentration of analytes during the sample preparation, for example by the application of solid phase extraction (SPE) [10,11]. Most importantly, the PA levels in samples had to be compared to reference standards that were analysed in PA-free matrix. Last but not least, the interference problem may be resolved by using tandem mass spectrometry applications (MS–MS) [12,13], single/multiple reaction monitoring technique (SRM/MRM) [12] or mass spectrometers with very high mass-accuracy and -resolution.

This manuscript describes the development of a matrix-specific, highly sensitive and reproducible ultra-performance liquid chromatography (UPLC) – high-resolution MS TOF method, which allows the trace analysis of PAs in *P. hybridus* extract Ze 339. Objectives of the development of the method were to achieve the simplest possible but exhaustive sample preparation, a fast and highly resolving UPLC separation system and a highly specific single-stage HRMS-time of flight (TOF) detection, which avoids interference with matrix substances as far as possible.

## 2. Materials and methods

### 2.1. Petasites hybridus Ze 339

For method development and validation, different lots of liquid *P. hybridus* extract Ze 339 were used, which were produced by $CO_2$-extraction of the dried and fine cut leaves of *P. hybridus* with a drug-extract ratio (DER) of 50–100:1. The extract presents itself as an oily viscose and almost clear liquid. The *P. hybridus* extract Ze 339 is registered for the treatment of allergic rhinitis in Switzerland and other countries and was provided by Max Zeller Söhne AG, Romanshorn, Switzerland. PAs were efficiently removed from the extract during extraction procedure by the use of a specific absorber technique.

### 2.2. Alkaloid reference standards

Senkirkine, senecionine, seneciphylline, senocionine-*N*-oxide, and seneciphylline-*N*-oxide used as external standards were obtained from PhytoLab GmbH & Co. KG, Germany. Purity was between 89% (senecionine-*N*-oxide), 98% (senkirkine) and >99% (senecionine, seneciphylline and seneciphylline-*N*-oxide).

### 2.3. Matrix solution/reference standards

In several available batches of *P. hybridus* extract, Ze 339 PA content was measured by this method. From these batches, one was chosen with non-quantifiable PA content and served as a basis for the preparation of matrix solution. To 1.5 g PA free *P. hybridus* extract Ze 339 2 mL of dichloromethane was given and mixed briefly using the test tube shaker. After addition of 4 mL of acetic acid 10% (v/v) and mixing for 10 min on an overhead shaker (30 rpm), the solution was centrifuged for 5 min at 5000 rpm (3885 × *g*). The upper aqueous phase was removed, and dichloromethane phase was extracted again with 4 mL of acetic acid 10% (v/v). The aqueous phases were unified and 1 mL of 25% ammonia solution (v/v) was added. Acetic acid 10% (v/v) was added up to a final volume of 10 mL. The prepared matrix solution was used for preparing the reference solutions. For this purpose, 10 mL of matrix solution was required per reference concentration. Internal and external reference standards were dissolved in methanol at concentrations of 10 μg/mL and 500 μg/mL, respectively. Reference standards were further diluted with acetic acid 10% (v/v) to achieve concentrations of 40, 125, and 300 ng/mL.

These dilutions were added to the prepared matrix solution to achieve matrix based reference standards at ten different concentration levels (0.3, 0.6, 1.3, 3.0, 6.3, 9.0, 12.5, 15.0, 22.5 and 30.0 ng/mL corresponding to 2.0, 4.0, 8.3, 20.0, 41.7, 60.0, 83.3, 100.0, 150.0 and 200.00 ppb).

### 2.4. Test solution

1.5 g *P. hybridus* extract Ze 339 was treated as described for matrix solution. The obtained solution was centrifuged over 5 min at 10,000 rpm (12,678 × *g*).

### 2.5. Liquid chromatography

A Waters Acquity H-Class UPLC-system (Waters, Milford, MA, USA) including a vacuum solvent degassing unit, a quarternary high pressure gradient pump, an automatic sample injector and a column thermostat were used. Chromatographic separation was achieved on an Acquity BEH C18, 1.7 μm, 75 × 2.1 mm column (Waters, Manchester, UK). The mobile phase consisted of 5 mM aqueous ammonium acetate buffer (pH 8.5) (A) and acetonitrile (B). The initial gradient condition was 98% A and 2% B linearly changed to 34% B over 8 min, followed by a step to 90% B until 8.10 min, held until 11.00 min and turned back to initial condition until 11.10 and washed by this until 15.00 min. The column temperature was adjusted at 50 °C. The flow rate was 0.6 mL/min and the injection volume was 4 μL.

### 2.6. Mass spectrometry

Mass spectrometry was performed in positive electrospray mode using a high resolution mass spectrometer synapt G2 S HDMS (Waters, Manchester, UK) with a TOF-detector with linear dynamic range of at least 5000:1, a mass resolution of minimum 30,000 FWHM (full peak width at half-maximum) at *m/z* 400 and a triple quadrupole with 4 kDa. The desolvation gas (45 °C, 800 L/h) and the nebulizer gas (6.0 bar) were nitrogen. The cone gas had a flow of 50 L/h. The capillary voltage was 0.50 kV and the source temperature 120 °C.

For the generation of mass spectra as for assessing, the identity of unknown PAs trap collision and transfer collision voltage was set at 4 V. The analyser mode was set at 'resolution' and the dynamic range at 'extended'. The mass spectra were acquired over the range of 50–600 Da with a spectral acquisition rate of 0.1 s per spectrum. Real time mass correction was performed using a solution of 0.5 ng/mL of leucine enkephalin ($C_{28}H_{37}N_5O_7$ at *m/z* 555.62).

### 2.7. Calculation

The content of each of PA was calculated from a calibration curve that was generated using the same alkaloid as an external standard

**Table 1**
Characteristics of used reference standards.

| Group | PA | Rt (min) | $[M+H]^+$ |
|---|---|---|---|
| Sencionine-type PA | Senecionine | 6.8 | 336.182 |
| | Senecionine-*N*-oxide | 4.2 | 352.176 |
| | Seneciphylline | 6.1 | 334.165 |
| | Seneciphylline-*N*-oxide | 3.5 | 350.160 |
| Otosenine-type PAs | Senkirkine | 4.5 | 366.191 |

Rt = retention time.

(see Table 1) using the TargetLynx software (Waters, Manchester, UK) as follows:

$$\mathrm{PA(ppb)} = \frac{(y-b)\times v}{m \times iw \times 1000}$$

where $y$ = area respectively area corrected by external standard; $b$ = ordinate intersection; $m$ = slope of standard curves; $V$ = sample volume (mL); 1000 = conversion factor from mg to μg; iw = initial weight in mg.

### 2.8. Validation

The assay was validated according to the ICH guideline of validation of analytical procedures [14].

The identity of PAs was assured by their retention time and mass (±0.001 Da) in comparison to the reference standards.

*Selectivity* was determined in blank matrix solution (see above).

*Recovery* was determined in five concentrations and 5 repeats.

*Intermediate precision* was analysed on two different levels (60 ppb and 150 ppb) by a second analyst.

*Accuracy* was tested at five concentrations (e.g. 2, 4, 20, 60, and 150 ppb; $N = 6$). At the lower two concentrations (e.g. 2 and 4 ppb) correlation coefficient (CV) limit was ≤25%, at the other concentrations ≤10%.

*Linearity* was shown for each external standards with 10 concentrations over a range of 2–200 ppb; $N = 3$. In order to minimize matrix effects, analysis of external standards was done in matrix solutions of PA-free *P. hybridus* extract.

*Limit of quantification* (LOQ) based on linearity data for each PA, the LOQ was defined at the lowest concentration of the standard concentration range.

*Robustness*: The stability of reference solution in PA free matrix was assessed after storing at room temperature and exposed to light for one week. The stability of test solutions was assessed at storage at 4 °C for 6 weeks. The acceptance criterion of ± 10% deviation related to the fresh prepared solutions was fulfilled for all reference PAs.

*System suitability test (SST)* was based on requirements of the Ph. Eur. Chapter 2.2.46 on different samples. The symmetry factor of the senkirkine peak and the coefficient of variation of repeated injections of a standard solution had to be between 0.8 and 1.5 and <3% ($N = 6$ injections), respectively.

**Table 2**
Recovery (%) of PAs at each of the quality control levels (ppb).

| PA | 2.0 ppb | 4.0 ppb | 20 ppb | 60 ppb | 150 ppb |
|---|---|---|---|---|---|
| Senecionine | 88.3 | 95.5 | 80.8 | 89.0 | 84.9 |
| Senecionine-*N*-oxide | 89.5 | 78.8 | 82.5 | 89.5 | 88.3 |
| Seneciphylline | 141.9 | 109.2 | 87.9 | 89.5 | 90.2 |
| Seneciphylline-*N*-oxide | 91.0 | 88.8 | 88.0 | 93.2 | 89.6 |
| Senkirkine | 108.3 | 109.0 | 89.8 | 94.3 | 98.3 |

**Table 4**
Inter-serial variability ($N = 6$). CV%.

| Levels | 60 ppb | 150 ppb |
|---|---|---|
| Senecionine | 1.33 | 2.20 |
| Senecionine-*N*-oxide | 5.31 | 1.48 |
| Seneciphylline | 1.22 | 1.79 |
| Seneciphylline-*N*-oxide | 1.62 | 2.47 |
| Senkirkine | 2.29 | 2.50 |

**Table 5**
Linearity (10 concentrations over a range of 2–200 ppb; $N = 3$).

| Standards | $R^2$ | Slope | Intercept | CV% |
|---|---|---|---|---|
| Senecionine | 0.99961 | 392.6 | 540.1 [a] | 7.4 |
| Senecionine-*N*-oxide | 0.99975 | 270.9 | 8.656 | 5.9 |
| Seneciphylline | 0.99948 | 366.1 | 383.0 [a] | 8.6 |
| Seneciphylline-*N*-oxide | 0.99961 | 237.8 | 0.465 | 7.5 |
| Senkirkine | 0.99949 | 304.8 | 314.8 [a] | 8.5 |

[a] Significant intercept.

## 3. Results and discussion

The structural formulas of internal and external standards of the PAs are shown in Fig. 1. Sample chromatograms of internal and external PA standards are given in Fig. 2. The identity of PAs was assured by their retention time and accurate mass (±0.001 Da) in comparison to the reference standards (Table 1).

*Selectivity* was determined with PA-free matrix. Retention times, and exact masses of the spiked samples were comparable with the References

Recovery (Table 2) over the whole range of tested concentrations was 80.8–95.5% for senecionine, 78.8–89.5% for senecionine-*N*-oxide, 141.9% for 2 ppb seneciphylline, 87.9–109.2% for 4–150 ppb, and 88.0–93.2% for seneciphylline-*N*-oxide. The higher recovery rate of seneciphylline at 2 ppb corresponds with other results on trace analysis [15]. The accuracy (Table 3) was lower than 5% with the exception of the two lower levels, where it was below 24%.

*Assessment of intermediate precision* showed that the variances of both series were homogeneous and the means were not significantly different. The coefficients of variation of the intermediate precision were within the acceptance criterion of <10%. Intra- and inter-serial variabilities were ≤10%. The results give first indication, that the method is precise and robust to a second analyst's influence (Tables 3 and 4).

**Table 3**
Mean accuracy (Repeatability) ($N = 6$). (ppb) / (CV%).

| | Nominal concentrations (ppb) | | | | |
|---|---|---|---|---|---|
| | 2 | 4 | 20 | 60 | 150 |
| Senecionine | 2.0/5.4 | 4.0/23.7 | 20.2/1.9 | 60.6/4.1 | 151.5/4.4 |
| Senecionine-*N*-oxide | 1.7/13.8 | 3.4/22.8 | 17.0/1.5 | 50.9/3.3 | 127.1/3.5 |
| Seneciphylline | 2.0/3.6 | 4.0/7.2 | 20.1/1.7 | 60.2/3.8 | 150.4/4.6 |
| Seneciphylline-*N*-oxide | 1.9/11.3 | 3.8/12.8 | 19.0/1.2 | 57.1/1.7 | 142.9/2.5 |
| Senkirkine | 1.9/5.4 | 3.7/23.7 | 18.7/1.9 | 56.2 / 4.2 | 140.5/2.5 |

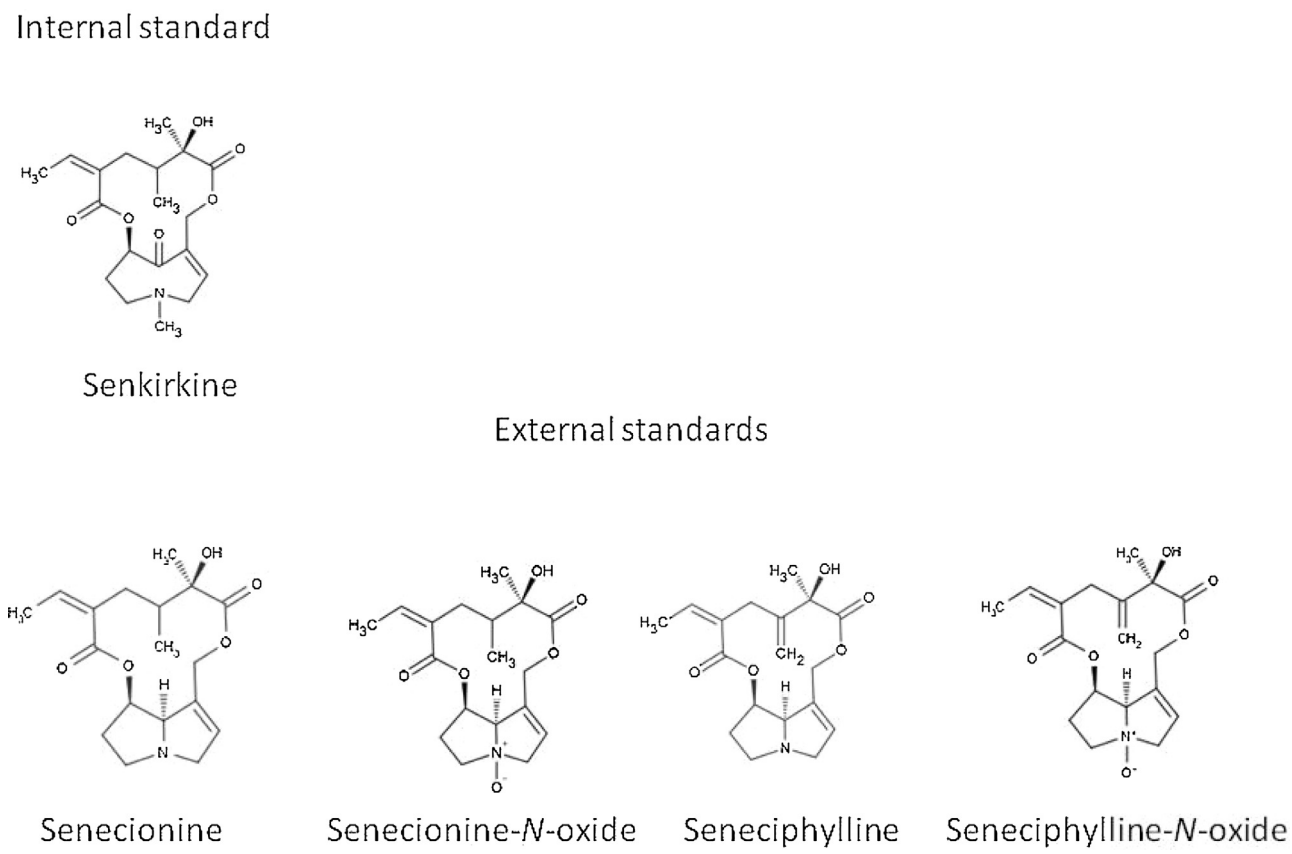


**Fig. 1.** Structural formulas of internal and external PAs.

*Linearity* was established in a concentration range of 2–200 ppb (Table 5). Statistical evaluation showed that for each PA linear regression was justified.

The five reference PAs in PA-free *P. hybridus* extract demonstrated a linear correlation within the concentrations from 0.3 to 30 ng/mL, defining the *working range* of the method. This approximately corresponds to a range of 2–200 ppb of each PA in a sample of *P. hybridus extract* Ze 339.

Based on linearity data for each PA, the LOQ was 2 ppb for each standard. The peaks at this concentration had been unambiguously identified, and quantification was accurate and reproducible (CV% ≤ 20).

Several methodological approaches have been discussed in the literature to reliably measure and quantify PAs in different biological matrices, by gas chromatography ([16–19]) and liquid chromatography with following mass spectroscopy ([8,10,17,18,20–30]).

Besides a lower sensitivity than LC–MS/MS methods ([17]), GC based methods have the principal drawback, that PA *N*-oxides are not directly determinable and require reduction to transform them into tertiary amines prior to determination. Thus, liquid chromatography (HPLC, UPLC) based methods coupled with HRMS techniques become more and more the standard in PA detection and quantification due to their high sensitivity and specificity.

Several other LC–MS methods for the determination of PAs, which were analysed in different matrices, have been published with a similar sensitivity [8,18,24,26,27,30,31]. However, all of them had only a low mass resolution, and most of them used MRM technique and/or determined LOQ in matrix free solution, only.

All robustness tests (see Table 6) fulfilled the condition of a prediction interval. This interval was calculated by the standard deviation of the repeatability precision.

**Table 6**
Parameters of robustness analysis (acceptance criterion).

| Parameters of robustness |
|---|
| UHPLC-conditions |
| Different batches of the stationary phase |
| Variation of the column temperature (± 5 °C) |
| Variation of the flow (± 0.05 mL/ min) |
| Variation of pH-value of the buffer (eluent A) |
| Variation of% eluent B (organic solvent) |
| MS-conditions |
| Variation of the capillary voltage (±2 kV) |
| Variation of the cone voltage (5 V) |
| Variation of the desolvation temperature (±50%) |

### 3.1. Selection of PA standards

In previous experiments (data not shown), we had used retrorsine and retrorsine-*N*-oxide as external standards to quantify PA content in matrix. However, these approaches were not successful. The different degrees of ionization of each PA, especially the substance specific impact of matrix effects on the ionization leading to different responses of the analytes, required the determination of specific correction factors for each PA. Compared with retrorsine, the same quantity of seneciphylline and senecionine gave approximately a two-fold different peak area. A correction factor of 0.51 has been calculated. With regard to senkirkine, this factor had to be set to 0.27. One difficulty was, however, that determination of these factors required the availability of the respective PA reference. For PAs, where no reference standard is available, quantification was not reliable. In those preliminary investigations, the lack of high mass accuracy of the MS led to false positive results. This effect was even more pronounced, the closer PA content was measured

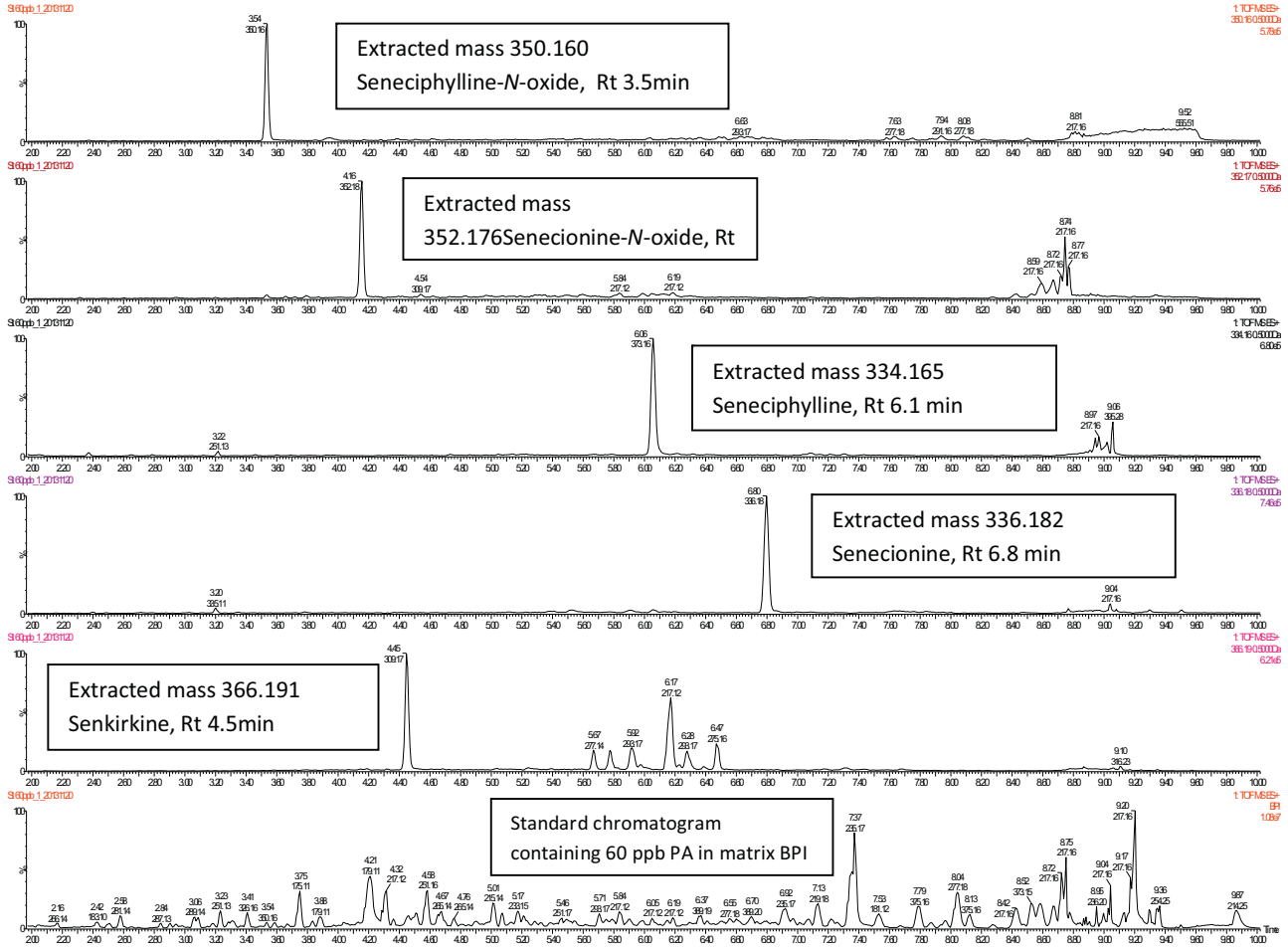


**Fig. 2.** Standard chromatograms of internal and external PAs showing retention times and exact masses ($[M+H]^+$). In addition, a chromatogram is given, where PAs were spiked to matrix base peak intensity (BPI).

to the LOQ. In order to identify PA reliably as such and to quantify them against a standard with the same response, it was decided to measure each PA against a reference substance of the same alkaloid. The use of high-resolution mass spectrometry, with additional fragmentation experiments and the consideration of retention times allowed a sufficiently reliable identification of PAs.

During validation, the possible advantage of using internal standards (retrorsine and retrorsine-*N*-oxide) was evaluated (Table 8). This analysis showed that an additional internal PA standard did not improve variability in linearity and precision assessment. Therefore, use of internal standards was omitted.

The industrial extraction process of *P. hybridus* extract Ze 339 was designed to completely remove PAs present in the dried leaves as raw material. Therefore, we had to use not properly purified extract batches, to identify, which alkaloids, present in the dried leaves of *P. hybridus,* might migrate from dried leaves into the liquid extract during the extraction process. Hereby, we could identify senkirkine, senecionine, seneciphylline, senocionine-*N*-oxide and seneciphylline *N*-oxide, which all were available as reference standards.

Since other PAs (such as 7– and 9-angeloylretronecine and their *N*-oxides, integerrimine and petasinine) were reported to be found in *P. hybridus* [8], the presence of these PAs was also investigated. This was done by selecting corresponding exact mass tracks in the chromatogram, by means of MS–MS or MRM experiments to investigate characteristic fragments corresponding to each of the different PAs. The characteristic fragments are for all retronecine type free-base PAs *m*/*z* 94, 120 and 138 [13] and for retronecine-type-*N*-oxides *m*/*z* 136 and 154 [32] and *m*/*z* 118 [23] in positive ionization mode. The PAs 7- and 9-angeloylretronecine-*N*-oxides were also available as qualitative reference substances. All of these studies showed that none of these PAs was present in the investigated not properly purified *P. hybridus* extract.

### 3.2. Matrix effects

It is well known that response in MS detection is significantly influenced by matrix effects [1]. Especially Electrospray Ionisation (ESI) is prone to be disturbed by simultaneously eluting matrix components. The most important interference is that the matrix components compete with the target analytes for the limited available surface charges. This leads to suppression of the formation of ions (ion suppression). This is especially relevant at higher concentrations, leading to falsely lower measured analyte levels [33]. In our method development of PA analysis for the *P. hybridus* extract Ze 339, a PA-free extract was available. This allowed measuring each of the reference standards in a matrix solution. Thereby, matrix effects could be largely avoided, because the matrix effects of the sample was allocated with the one of the reference solutions.

The significance of these effects was demonstrated by the comparison of raw material (leaves) from *P. hybridus* and other plant material as matrices. In comparison with the *P. hybridus* extract Ze 339 for PA determination in *P. hybridus* leaves, no alkaloid free matrix material was available. Therefore, preparations from other (alkaloid free) plants served as a substitute matrix [34]. The effect of different matrices is exemplified in Table 7: Three dif-

**Table 7**
PA-standards and their recovery in aqueous solution, matrix solution from artichoke leaves and matrix solution from *P. hybridus* root.

| PA | Weighted standard(ng/mL) | Recovery (%) | | |
|---|---|---|---|---|
| | | Aqueous solution | Artichoke matrix | *P. hybridus* matrix |
| Senkirkine | 28.10 | 99.0 | 33.6 | – |
| Seneciphylline | 30.08 | 98.9 | 24.4 | – |
| Senecionine | 30.31 | 97.5 | 20.8 | – |
| Senecionine-*N*-oxide | 25.43 | 98.3 | 30.6 | – |
| Seneciphylline-*N*-oxide | 28.57 | 99.4 | 24.8 | – |
| Monocrotaline | 30.73 | 97.0 | 15.1 | 27.2 |
| Monocrotaline-*N*-oxide | 31.60 | 96.0 | 34.0 | 28.5 |
| Intermedine | 33.77 | 98.2 | 19.2 | 31.9 |
| Lycopsamine | 29.59 | 98.1 | 18.7 | 31.8 |
| Retrorsine | 25.64 | 113.0 | 25.7 | 31.2 |
| Retrorsine-*N*-oxide | 25.04 | 96.9 | 30.6 | 29.3 |
| Heliotrine | 28.96 | 99.7 | 31.4 | 35.7 |
| Heliotrine-*N*-oxide | 29.02 | 98.3 | 33.1 | 35.1 |
| Echimidine | 27.93 | 98.5 | 34.0 | 40.1 |

**Table 8**
Effect of the use of internal standards (ISTD) on the variability of the assessment of linearity and precision.

| | Linearity | | | | Precision at 20 ppb level | |
|---|---|---|---|---|---|---|
| PA | r without ISTD | r with ISTD | RSD without ISTD | RSD with ISTD | RSD without ISTD | RSD with ISTD |
| Senkirkine | 0.99968 | 0.99970 | 2.65 | 2.56 | 1.94 | 2.53 |
| Senecionine | 0.99987 | 0.99976 | 1.73 | 2.35 | 3.20 | 2.72 |
| Senecionine-*N*-oxide | 0.99989 | 0.99994 | 1.58 | 1.18 | 1.53 | 2.18 |
| Seneciphylline | 0.99960 | 0.99942 | 3.02 | 3.65 | 1.67 | 2.72 |
| Seneciphylline-*N*-oxide | 0.99964 | 0.99962 | 2.80 | 2.89 | 1.24 | 1.72 |

*r* = regression coefficient, ISTD internal standard, RSD = relative standard deviation.

ferent matrices (aqueous solution, artichoke and the *P. hybridus* leave matrix) were spiked with PAs, which do not naturally occur in the plant. These experiments showed firstly, the significant ion suppression by the presence of matrix components in general (the recovery remained partly below one third of the absolute input of the reference substances), and, secondly, the different extend of ion suppression when using different matrices.

This suggests that using reference standards dissolved in one standard plant mixture matrix for the analysis of different other plants (such as described in [34]) or dissolved in a matrix-free solution (such as in [8]) will result in a significant, systematic bias of PA quantification.

We analysed 29 different batches of the *P. hybridus* extract Ze 339 for their PA content. None of the tested batches contained quantifiable (e.g. ≥2 ppb) concentrations of unsaturated PAs.

In summary, we have developed a highly sensitive UPLC MS TOF method with a limit of quantification of 2 ppb for all naturally occurring PAs. Recovery at the LOQ was between 88.9 and 141.9%, the repeatability precision between 3.5 and 13.6%. Linearity of the five PAs showed correlation coefficients between 0.9995 and 0.9998 and coefficients of variation between 7.44 and 8.56%. A working range between 2 ppb and 200 ppb could be fixed. It may be concluded that this assay allows to reliably determine trace PA concentrations in *P. hybridus* extract Ze 339, making it suitable for analytical PA monitoring in accordance with regulatory requirements.

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