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The Analysis of Pyrrolizidine Alkaloids in *Jacobaea vulgaris*; a Comparison of Extraction and Detection Methods

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ABSTRACT:
Introduction – Pyrrolizidine alkaloids (PAs) serve an important function in plant defence.
Objective – To compare different extraction methods and detection techniques, namely gas chromatography with nitrogen phosphorus detection (GC-NPD) and liquid chromatography tandem mass spectrometry (LC-MS/MS) with quadrupole analysers for analysing PAs in *Jacobaea vulgaris*.
Methodology – Both formic acid and sulfuric acid were tested for PA extraction from dry plant material. For GC-NPD, reduction is required to transform PA *N*-oxides into tertiary amines. Zinc and sodium metabisulfite were compared as reducing agents.
Results – The lowest PA concentration measured with GC-NPD was approximately 0.03 mg/g and with LC-MS/MS 0.002 mg/g. The detection of major PAs by both techniques was comparable but a number of minor PAs were not detected by GC-NPD. With the LC-MS/MS procedure higher concentrations were found in plant extracts, indicating that losses may have occurred during the sample preparation for the GC-NPD method. Zinc proved a more effective reducing agent than sodium metabisulfite. The sample preparation for LC-MS/MS analysis using formic acid extraction without any reduction and purification steps is far less complex and less time consuming compared to GC-NPD analysis with sulfuric acid extraction and PA *N*-oxide reduction with zinc and purification.
Conclusions – In terms of sensitivity and discrimination, formic acid extraction in combination with LC-MS/MS detection is the method of choice for analysing PAs (both free and *N*-oxides forms) in plant material.

Keywords: gas chromatography; *Jacobaea vulgaris* (*Senecio jacobaea*); liquid chromatography tandem mass spectrometry; pyrrolizidine alkaloids; reduction; secondary metabolites; Tansy ragwort

Introduction

Pyrrolizidine alkaloids (PAs) are regarded as chemical defence substances against herbivores (Bentley *et al.*, 1984; Dreyer *et al.*, 1985; Vrieling *et al.*, 1991; Leiss *et al.*, 2009) and pathogenic fungi (Hol and Van Veen, 2002). Plants containing PAs such as *Jacobaea vulgaris* Gaertn. (also known as *Senecio jacobaea*) are an important food source for numerous insect species (Harper and Wood, 1957). *Jacobaea vulgaris* originates from Europe and Asia (Cameron, 1935; Harper and Wood, 1957) and was introduced into North America, New Zealand and Australia (Pemberton and Turner, 1990). In the introduced areas it is considered a pest species as it causes extensive livestock losses and is a potential contaminant in milk and honey. When parts of these plants are ingested in substantial quantities, PAs can cause acute and chronic liver damage in mammals, including humans. It is therefore of great importance to develop analytical methods including sample preparation, extraction and detection techniques to get a better insight in the physiology, ecology and toxicology of PA containing plants. The mode of action in invertebrate species is not well known, but Frei *et al.* (1992) showed that PAs caused DNA damage. In general PAs deter generalist herbivores (Van Dam, 1995; Macel *et al.*, 2005); however several specialist herbivores have adapted to PAs in their diet. In fact some specialists even prefer plants containing PAs (Boppré, 1986; Hartmann and Witte, 1995; Macel and Klinkhamer, 2009).

PAs occur in free (tertiary amine) or *N*-oxide forms. Until now it has been generally assumed that in plants PAs are mainly present as *N*-oxide except in some seeds. However, recently we found that some PAs of *J. vulgaris* also occur in substantial amounts as tertiary amine (unpublished data). This is of importance for understanding the role of PAs as plant defence

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compounds, as the tertiary PA form is known for its strong negative influence on several plant attackers. The *N*-oxide form is considered to be non-toxic (Lindigkeit *et al.*, 1997).

For many years, PAs were typically isolated by acid–base extraction in combination with zinc reduction (Hartmann and Zimmer 1986; Witte *et al.*, 1993). Gas chromatography (GC) with flame ionisation detection (FID) or nitrogen phosphorus detection (NPD) have been typically used as analytical methods (Hartmann and Toppel, 1987; Witte *et al.*, 1992; Hartmann and Dierich, 1998; Kowalchuk *et al.*, 2006). Over the years several other PA extraction methods (Pieters *et al.*, 1989; Betteridge and Colegate, 2005; Joosten *et al.*, 2009; Jiang *et al.*, 2009) and techniques for analysing PAs have been applied such as spectrophotometry (Mattocks, 1967; De Boer, 1999), TLC, HPLC-UV, GC-MS (Hartmann and Toppel, 1987; Witte *et al.*, 1992; Hartmann and Dierich, 1998) and NMR (Pieters *et al.*, 1989, Leiss *et al.*, 2009). Recently liquid chromatography–tandem mass spectrometry (LC-MS/MS) has been introduced for measuring PAs in plant material (Wuilloud *et al.*, 2004; Betteridge and Colegate, 2005; Zhang *et al.*, 2008; Jiang *et al.*, 2009; Joosten *et al.*, 2009). Unlike GC-related methods, LC-MS/MS and NMR can detect both tertiary amines and *N*-oxides without the time-consuming and tedious reducing step.

The aim of this study was to compare two different methods for the analysis of PAs; the traditional method consisting of sulfuric acid extraction, reduction of PA *N*-oxide with zinc and then purification by liquid–liquid extraction followed by GC-NPD analysis. The other LC-MS/MS method involves a formic acid extraction without reduction and further purification steps. In addition we also compared some critical steps involved in the two methods (Fig. 1). For example we investigated whether, irrespective of the sample preparation procedure, detection of (reduced) PAs with GC-NPD and LC-MS/MS (procedures 4, 6, 8 vs 5, 7, 9) give similar results with respect to the concentration and composition in plant material. Furthermore, we tested the efficiency of formic acid extraction as an alternative to sulfuric acid extraction (procedures 6, 7 vs 8, 9). We also evaluated another reducing agent, sodium metabisulfite ($Na_2S_2O_5$), as an alternative to the commonly used zinc dust (procedures 2 vs 3 and 4, 5 vs 6, 7) for GC-based analysis. Finally we investigated whether the traditional and the novel analytical methods give similar results with regards to PA concentration and composition in plant material (procedure 1 vs 8).

Materials and Methods

Plant material samples

Five dried *J. vulgaris* samples were used for PA analysis. One of the samples was used as a reference as it contained a mixture of several different *J. vulgaris* plants (aerial parts) collected in Meijendel (The Netherlands) in July, 1997. This sample had been used in the past for identification of PAs detected by GC-NPD. The other four samples (two roots and two aerial parts) were made from a genotype that originated from a dune population in 'Meijendel' in our tissue culture laboratory. Two plants were propagated from the genotype by tissue culture and grown for 6 weeks in a climate room (relative humidity 70%, light 16 h at 20°C, dark 8 h at 20°C). After 6 weeks the two plants were harvested and cut above the root crown by scissors. The plants were divided into roots (R1 and R2) and aerial parts (S1 and S2) and freeze-dried for 72 h under vacuum with a collector temperature of −55°C (Labconco Free Zone® 12 l Freeze Dry System, Kansas City, MO, USA).

Pyrrolizidine alkaloid analysis

Formic acid extraction (procedures 1–7). Ten milligrams of dried and ground plant material was extracted with 2% formic acid in a 1.2:100 w/v ratio. The plant extract solution was shaken for 1 h. Solid plant material was removed by centrifugation at 2600 rpm for 10 min. Heliotrine (100 µg/mL in methanol) was added as an internal standard to a concentration of 1 µg/mL. An aliquot of the extract (25 µL) was diluted with water (975 µL) and injected in the LC-MS/MS system (procedure 1 in Fig. 1).

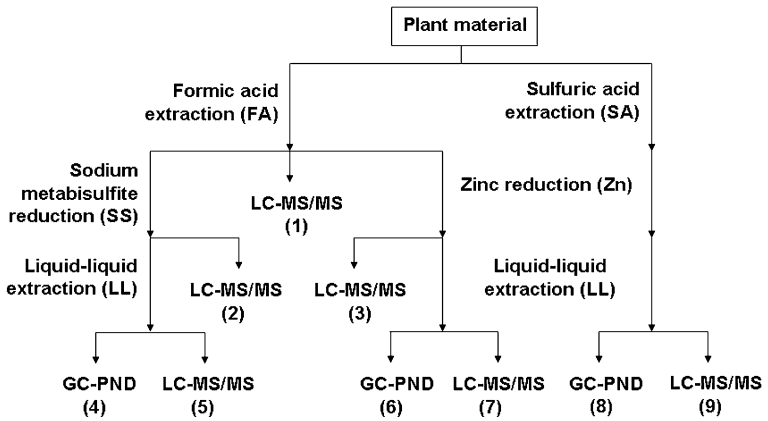

Figure 1. Extraction and detection of PAs from dried plant material and the different procedures applied. Dry plant material was extracted with formic acid (1–7) or sulfuric acid (8–9). The formic acid extract was either directly measured on the LC-MS/MS (1) or reduced with sodium metabisulfite (2, 4, 5) or reduced with zinc (3, 6, 7). The sulfuric acid extract was only reduced with zinc (8–9). The reduced extracts were purified by liquid–liquid extraction and analysed with GC-NPD (4, 6, 8) or LC-MS/MS (5, 7, 9). Reduced extracts were also analysed directly by LC-MS/MS before liquid–liquid purification (2–3). Procedure 8 is the traditional method and procedure 1 the relatively novel method.

Sulfuric acid extraction with zinc reduction (procedures 8 and 9). Ten milligrams of dried and ground plant materials was extracted with 0.25 M sulfuric acid (H_2SO_4) in a 1.5:100 w/v volume ratio. The plant extract solution was shaken for 1 h and subsequently the plant material was removed by centrifugation at 2600 rpm for 10 min. Zinc dust was added to the extraction solution and shaken for 2 h to reduce the PA *N*-oxides. Sulfuric acid extraction and zinc reduction (procedures 8 and 9 in Fig. 1) were performed following the 'standard' extraction method of Hartmann and Zimmer (1986) and adapted for small quantities by De Boer (1999) for GC analysis.

Sodium metabisulfite reduction (procedures 2, 4 and 5). One molar sodium metabisulfite was added to the formic acid extract solution in a 1:80 v/v ratio and shaken for 2 h to reduce the PAs *N*-oxides. At this stage an aliquot was taken for LC-MS/MS analysis (25 µL), diluted with water (975 µL) and heliotrine (100 µg/mL in methanol) was added as an internal standard to a concentration of 1 µg/mL (procedure 2 in Fig. 1).

Zinc reduction (procedures 3, 6 and 7). A small 'knife tip' of zinc dust was added to the formic acid extract solution and shaken for 2 h to reduce the PAs *N*-oxides. If during shaking no visible hydrogen bubbles were formed anymore, additional zinc was added. At this stage an aliquot (25 µL) was diluted with water (975 µL) and after addition of heliotrine (100 µg/mL in methanol) as an internal standard to a concentration of 1 µg/mL (procedure 3 in Fig. 1), it was analysed by LC-MS/MS.

Purification by liquid-liquid extraction (procedures 4–9). To the reduced extract, 25% ammonium hydroxide was added to reach a pH 9–10. The extract was purified by applying it over a 0.60 × 30 mm Extrelut® NT 1 column (Merck, Darmstadt, Germany). The column was eluted with dichloromethane. After elution the dichloromethane fraction was evaporated overnight and the residue was redissolved in 150 µL methanol (MeOH) containing 0.5 mg/mL heliotrine as internal standard.

Analysis by LC-MS/MS (procedures 1–3, 5, 7 and 9). The aqueous extracts obtained from procedures 1–3 were analysed. From the methanolic extracts obtained after liquid–liquid purification (procedures 5, 7 and 9 in Fig. 1) aliquots (10 µL) were diluted with water (990 µL) and injected into the LC-MS/MS system. The LC-MS/MS system consisted of an Agilent HP1100 HPLC equipped with a binary pump system, an autoinjector and a column oven, coupled to a Waters Micromass Micro tandem mass spectrometer.

Chromatographic separation was achieved on a Waters Xbridge 150 × 3.0 mm HPLC column, run with a water–acetonitrile linear gradient containing 0.05% ammonia (pH 11 ± 0.5) at a flow rate of 0.4 mL/min. The gradient started at 100% water (2 min) and during analysis the acetonitrile percentage was raised to 70% in 16 min. The column was kept at 40°C and the injection volume was 5 µL.

An electrospray ion source of the quadrupole mass analyser was used in positive mode, with the following instrument settings: capillary voltage, 2.7 kV; source temperature, 100°C; cone gas (N_2) flow, 50 L/h; desolvation gas (N_2) flow, 600 L/h; desolvation temperature, 400°C. Argon was used as collision gas at a pressure of 3.0×10^{-3} mbar. Data were recorded in multiple monitoring mode (MRM) using one selected precursor to product ion transition per compound. Based on the retention times of the individual PAs the transitions could be combined into three MRM windows. One window contained the early eluting PAs, and a second window the late eluting PAs. The PAs with precursor mass *m/z* 352 were combined into a third window, because they overlapped with the other two windows. In this way the number of simultaneously monitored transitions (channels) could be limited

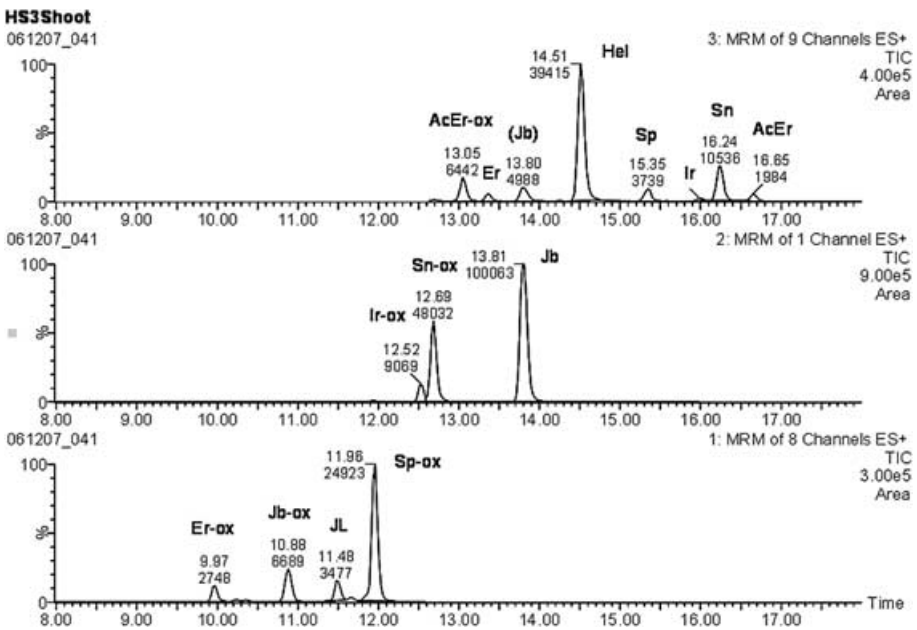

Figure 2. LC-MS/MS chromatograms of pyrrolizidine alkaloids in a *Jacobaea vulgaris* aerial part extract. The various precursor to product ion transitions (channels) have been combined in this figure into three MRM windows. Mass spectrometer settings and abbreviations of individual PAs are as explained in Table 1. Some of the minor PAs are not visible in the combined MRM chromatograms. These PAs can be seen when the individual transitions are plotted (data not shown).

Table 1. MS/MS fragmentation conditions and LC retention times for PA identified in *J. vulgaris* extracts. The mean PA concentration determined with the LC-MS/MS method

Pyrrolizidine alkaloid	Abbreviation	Precursor to product ion transition (m/z)	Cone voltage (V)	Collision Energy (V)	Retention time (min)	Mean PA concentration (µg/g)	Occurrence in samples
Heliotrine	Hel	314.2 > 138.0	30	25	14.51		
Seneciphylline	Sp	334.2 > 120.0	40	30	15.35	56.3	5/5
Seneciphylline *N*-oxide	Sp-ox	350.2 > 120.0	40	30	11.96	398.2	5/5
Spartioidine[a]	St	334.2 > 120.0	40	30	15.10	1.1	2/5
Integerrimine	Ir	336.2 > 120.0	40	30	16.00	13.2	5/5
Integerrimine *N*-oxide	Ir-ox	352.2 > 120.0	40	30	12.52	153.6	5/5
Senecionine	Sn	336.2 > 120.0	40	30	16.24	90.9	5/5
Senecionine *N*-oxide	Sn-ox	352.2 > 120.0	40	30	12.69	923.7	5/5
Jacozine[a]	Jz	350.2 > 94.0	40	40	13.19	7.6	5/5
Erucifoline[a]	Er	350.2 > 94.0	40	40	13.39	12.8	5/5
Erucifoline *N*-oxide[a]	Er-ox	366.2 > 94.0	40	40	9.97	33.3	5/5
Riddelliine	Rd	350.2 > 94.0	40	40	13.78	1.9	5/5
Riddelliine *N*-oxide	Rd-ox	366.2 > 94.0	40	40	10.83	21.4	5/5
Jacobine	Jb	352.2 > 120.0	40	30	13.81	482.5	5/5
Jacobine *N*-oxide	Jb-ox	368.2 > 94.0	40	40	10.88	600.7	5/5
Retrorsine	Rt	352.2 > 120.0	40	30	14.44	1.4	3/5
Retrorsine *N*-oxide	Rt-ox	368.2 > 94.0	40	40	11.53	3.15	2/5
Jacoline[a]	Jl	370.2 > 120.0	40	30	11.50	21.1	5/5
Jacoline *N*-oxide[a]	Jl-ox	386 > 94.0	40	40	9.20	9.6	5/5
Acetylseneciphylline	AcSp	376.2 > 120.0	40	30	18.54	6.0	5/5
Acetylseneciphylline *N*-oxide	AcSp-ox	392.2 > 120.0	40	30	14.43	6.7	5/5
Jaconine[a]	Jn	388.2 > 120.0	40	30	14.82	20.0	5/5
Jaconine *N*-oxide[a]	Jn-ox	404.2 > 94.0	40	40	11.14	5.3	3/5
Acetylerucifoline[a]	AcEr	392.2 > 120.0	40	40	16.65	8.1	5/5
Acetylerucifoline *N*-oxide[a]	AcEr-ox	408.2 > 94.0	40	40	13.05	38.9	5/5

[a] No purified standard available. Settings were optimised using a plant extract of *Jacobaea vulgaris*.

to 10 (dwell time 120 ms/transition) (Fig. 2). Cone and collision energy settings were optimised for the individual compounds (Table 1). Obtained peak areas were internally calibrated using the internal standard heliotrine and the individual compounds were quantified against a standard solution of the PAs in water. Fourteen individual PA standards were available for this study, representing over 90% of the total amount of PAs present in the plant extracts. The remaining PAs, being tertiary amines as well as *N*-oxides, were quantified by using the mean response of the tertiary amine standards and the *N*-oxide standards, respectively. Data processing was conducted with Masslynx 4.0 software (Waters, Milford, MA, USA).

Analysis by GC-NPD (procedures 4, 6 and 8). The methanol extracts of procedures 3, 6 and 8 (Fig. 1) were determined with GC-NPD (Hewlett Packard 6890 and a 30 m × 0.25 µm, HP-1) under the following conditions: injector 250°C, temperature programmed from 220°C (3 min) to 250°C at 5°C/min, in the split mode (1 : 20), carrier gas flow (N_2) 0.9 mL/min, pressure 560 mbar, detector NPD. The injection volume was 1 µL. Quantitative analysis were performed via the NPD signals using heliotrine as internal standard.

Data processing was conducted with Microsoft Office Excel 2003 for Windows (Microsoft Corporation, Redmond, WA, USA). For identification of the tertiary PAs, retention times were compared with a reference sample (Table 2). The reference sample ('M') contained a mix of dried aerial part material of several different *J. vulgaris* plants collected in 'Meijendel'.

Data analysis. Data were all normally distributed. The correlation between the PA concentration measured by GC-NPD and LC-MS/MS was tested by the Pearson correlation coefficient with SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA). Regression was based on 15 data points (5 samples × 3 different extraction methods). The regression coefficient was estimated by linear regression model II (Sokal and Rohlf, 1981; de Jong and Klinkhamer, 2005). We used 'two-factor ANOVA without replication' to analyse if the different methods and techniques did have a significant influence on the estimates the total PA concentration and the estimates of the concentration of different major PAs. Regression and 'two-factor ANOVA without replication' were conducted with Microsoft Office Excel 2003 for Windows (Microsoft Corporation, Redmond, WA, USA).

Table 2. GC-NPD retention time of individual PAs and the mean PA concentration (tertiary amines + reduced *N*-oxides) in *J. vulgaris* extracts, determined with the traditional method

Pyrrolizidine alkaloid	Abbreviation	Retention time (min)	Mean PA concentration (μg/g)	Occurrence in samples
Heliotrine internal standard	Hel	5.4		
Senecionine	Sn	7.3	485.9	5/5
Seneciphylline	Sp	7.6	282.1	5/5
Integerrimine	Int	8.1	92.1	5/5
Jacobine	Jb	9.5	433.4	5/5
Jacozine	Jz	9.9	23.9[a]	2/5
Jacoline	Jl	10.4	55.2[a]	1/5
Erucifoline	Er	11.0	8.7[a]	2/5

[a] Concentration may be an underestimate, because in some samples the amount could not be quantified.

Results and Discussion

Detection of PAs by GC-NPD and LC-MS/MS

The GC-NPD and LC-MS/MS detection techniques for the determination of PAs in plant material were compared by the analysis of a number of extracts of dried *J. vulgaris* (procedures 4, 6, 8 vs 5, 7, 9). In this study the lowest PA concentration in dry plant material that could be quantified with GC-NPD was approximately 30 μg/g and with LC-MS/MS around 2 μg/g. The introduction of a tandem mass spectrometer as a detector in combination with liquid chromatographic separation thus greatly improves the possibilities of determination of individual PAs by lowering the detection limits (Betteridge and Colegate, 2005; Wuilloud *et al.*, 2004). It should be noted that, for LC-MS/MS analysis, the plant extracts had to be diluted 40 times, to keep the peak responses of the most abundant PAs within the linear dynamic range of the mass spectrometer, while for GC-NPD analysis the final extracts had to be concentrated approximately four times. As a result LC-MS/MS detected up to 13 different tertiary PAs in *J. vulgaris* extracts while GC-NPD detected only seven PAs (Tables 1 and 2). With GC-NPD only four major PAs (senecionine, seneciphylline, integerrimine and jacobine) were detected in all samples (Fig. 3). The concentrations of the other three PAs were sometimes just below the quantitation limit of the GC-NPD (Table 2).

Omission of the reduction step resulted in the additional detection of 11 different PA *N*-oxides by LC-MS/MS. Thus the number of PAs detected is almost doubled. It is a major advantage of LC-MS/MS that it can determine both *N*-oxides and tertiary amines directly, without the necessity of reduction of *N*-oxides to the corresponding tertiary amines, as is required for GC-based methods.

GC-NPD and LC-MS/MS measurements of the reduced extracts (procedures 4–9) were found to be highly correlated with respect to the total PA concentration ($r^2 = 0.98$) as well as the four major individual PA concentrations separated (Fig. 4).

Formic acid vs sulfuric acid as extraction solvent

Formic acid is often used in analytical methods with LC-MS detection. Formic acid forms volatile salts when combined with a mobile phase that contains ammonia as modifier. Sulfuric acid, on the other hand, will form non-volatile salts that can precipitate in the source of the mass detector, and in the long run will affect sensitivity and reproducibility. For this reason direct injection of (diluted) sulfuric acid extracts into the LC-MS/MS system is not recommended. The formic acid and sulfuric acid extractions were compared (procedures 6 vs 8 and 7 vs 9 in Figs 1 and 5). Exchange of sulfuric acid as extraction solvent by formic acid did not lead to significant differences in concentration of the total PAs or the individual major PAs when measured with GC-NPD or LC-MS/MS.

Zinc vs sodium metabisulfite as reduction method

Reduction of PA *N*-oxides with zinc dust is commonly used when the analysis is performed by GC based methods. Hartmann and Toppel (1987) described the use of sodium dithionite ($Na_2S_2O_4$) in sulfuric acid as alternative to zinc as reducing agent. We observed that reduction with sodium dithionite in combination with formic acid proceeded very slow. As an alternative we tested sodium metabisulfite ($Na_2S_2O_5$). A 10 mM solution of sodium bisulfite in dilute formic acid efficiently reduced PA *N*-oxides. To compare sodium bisulfite reduction with zinc, the total PA

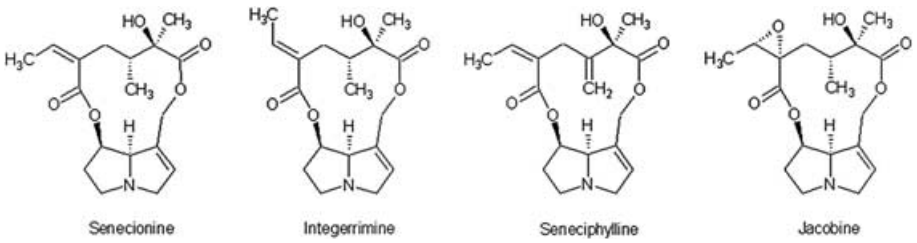

Figure 3. The chemical structures of tertiary amine forms of four major PAs present in *Jacobaea vulgaris* extract. **N*-oxide forms: N → O.

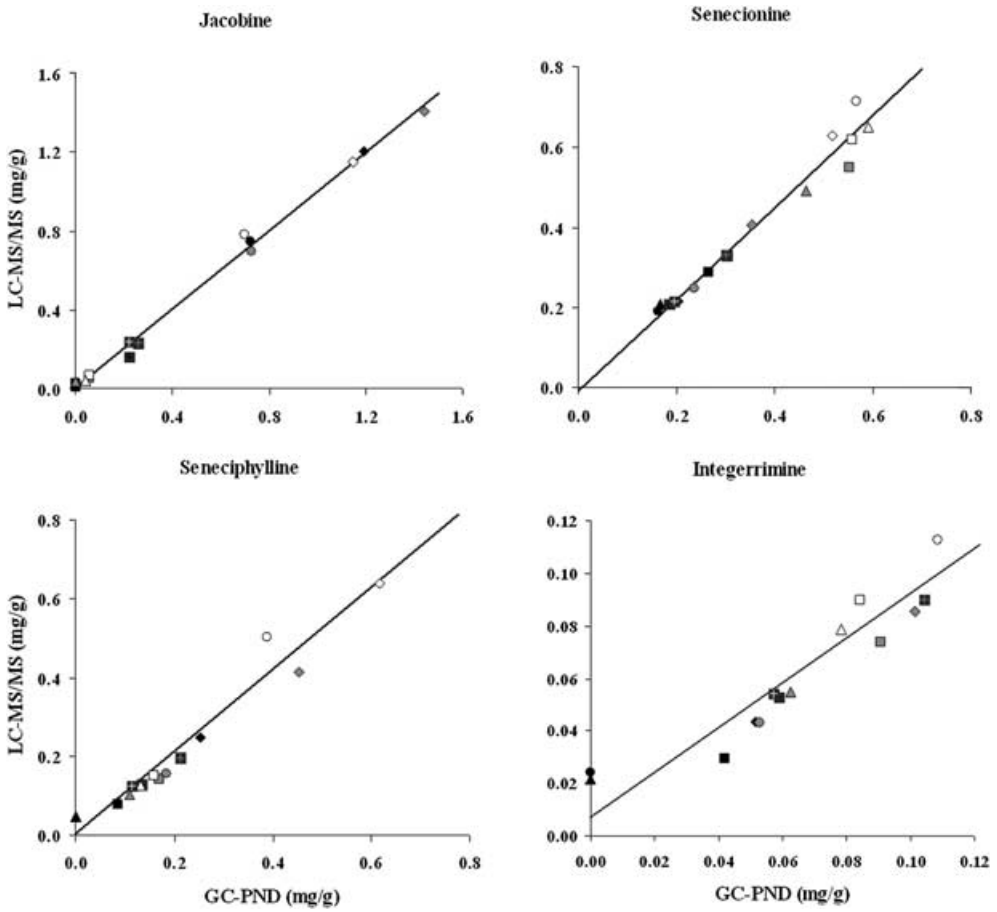

Figure 4. Relationship between the concentrations of jacobine ($y = 0.995x + 0.006$, $r^2 = 0.996$, $p < 0.01$, $n = 15$), senecionine ($y = 1.146x - 0.008$, $r^2 = 0.972$, $p < 0.01$, $n = 15$), seneciphylline ($y = 1.057x - 0.009$, $r^2 = 0.955$, $p < 0.01$ $n = 15$) and integerrimine ($y = 0.854x + 0.007$, $r^2 = 0.904$, $p < 0.01$, $n = 15$) concentration (mg/g dry plant material) in samples analysed by GC-NPD and LC-MS/MS. Fifteen different points represent five samples (■ = root 1, ◆ = aerial part 1, ▲ = root 2, ● = aerial part 2 and + = reference sample) and three extraction/reduction methods (black = 4 and 5, grey = 6 and 7 and white = 8 and 9; Fig. 1).

concentration of formic acid extraction before purification was measured with LC-MS/MS (procedure 2 vs 3 in Fig. 1). Zinc reduction gave a higher total PA concentration compared with sodium metabisulfite, respectively 1.96 and 1.43 mg/g dry plant material ($F_{1,4} = 2.1$, $p = 0.224$), but this was not significant due to the high levels of jacobine in the two aerial part samples (Fig. 6a). For jacobine no significant difference was found between the two reducing agents ($F_{1,4} = 0.7$, $p = 0.451$). The concentrations of other major PAs nearly doubled when zinc was used instead of sodium metabisulfite, respectively for senecionine 1.9 and 1.2 mg/g ($F_{1,4} = 11.7$, $p < 0.05$), seneciphylline 0.4 and 0.2 mg/g ($F_{1,4} = 16.4$, $p < 0.05$) and integerrimine 0.15 and 0.08 mg/g ($F_{1,4} = 22.6$, $p < 0.01$) dry plant material.

A similar result was obtained when the total PA concentration among the three different extraction/reduction combinations (procedures 4–5, 6–7 and 8–9) was compared (Fig. 5). Formic acid extraction in combination with sodium bisulfite reduction resulted in the lowest mean total PA concentration, respectively 0.78 and 0.81 mg/g dry plant material according to GC-NPD and LC-MS/MS. Sulfuric acid extraction with zinc reduction produced the highest mean total PA concentration, 1.29 and 1.42 mg/g dry plant material, respectively. It can be concluded that on the whole zinc dust is somewhat more efficient in transforming PA *N*-oxides to the corresponding tertiary amines.

Comparison between GC-NPD and LC-MS/MS method

Sulfuric acid extraction in combination with zinc reduction of the *N*-oxides followed by liquid–liquid purification and GC-NPD analysis can be regarded as the traditional method of PA analysis (procedure 8 in Fig. 1). Formic acid extraction without reduction and purification steps, followed by LC-MS/MS detection is the newer method (procedure 1 in Fig. 1). For comparison of the LC-MS/MS data with those of the GC-NPD, the individual PA concentrations of the *N*-oxides and tertiary amines obtained for the LC-MS/MS samples were summed.

The LC-MS/MS method gave a significantly higher total PA concentration compared with the traditional method (Fig. 6b), 2.18 and 1.29 mg/g dry plant material ($F_{1,4} = 10.7$, $p < 0.05$), respectively. For jacobine no significant difference was found between the two methods ($F_{1,4} = 4.4$, $p = 0.105$), but for the other three major PAs the concentration nearly doubled using the LC-MS/MS method. It should be noted that this difference was only a trend close to significance, for senecionine 0.49 and

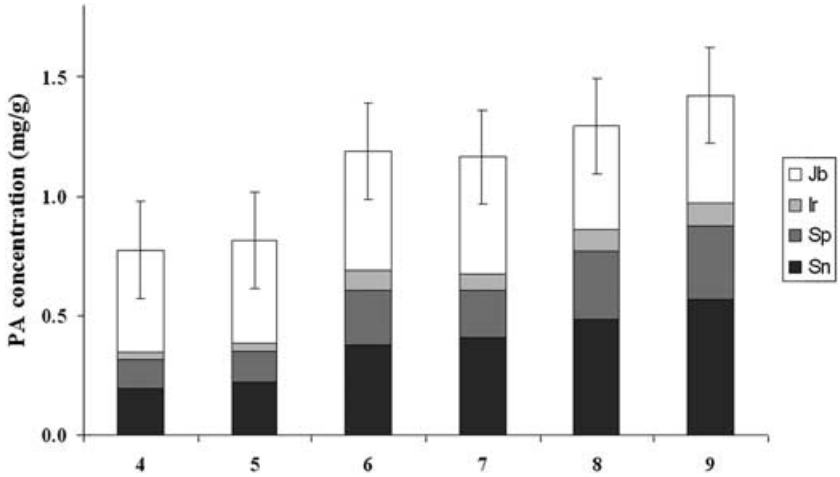

Figure 5. The mean PA concentration (±SE-bar of total PA concentration) of formic acid extracts reduced by zinc analysed with GC-NPD (4) or LC-MS/MS (5). Formic acid reduced by sodium metabisulfite with GC-NPD (6) or LC-MS/MS (7). Sulfuric acid extracts reduced with zinc analysed with GC-NPD (8) or LC-MS/MS (9).

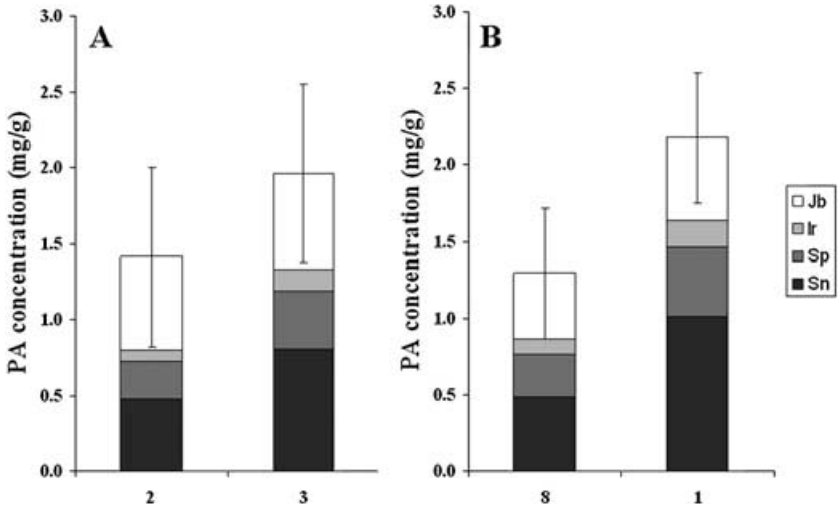

Figure 6. (A) The mean PA concentration (±SE-bar of total PA concentration) analysed with LC-MS/MS of formic acid extracts reduced by sodium metabisulfite (2) and by zinc (3). (B) The mean PA concentration obtained with the traditional method: sulfuric acid extraction, PA *N*-oxide reduction with zinc, purification and GC-NPD analysis (8) and the newer method: formic acid extraction with LC-MS/MS analysis (1).

1.01 mg/g ($F_{1,4} = 6.0$, $p = 0.07$), seneciphylline 0.28 and 0.45 mg/g ($F_{1,4} = 7.7$, $p = 0.05$) and integerrimine 0.09 and 0.17 mg/g ($F_{1,4} = 6.3$, $p = 0.07$) were obtained in dry plant material, respectively.

The PA concentrations obtained by the two different extraction methods (formic acid vs sulfuric acid) and the two detection techniques (GC-NPD vs LC-MS/MS) were more or less comparable as described above. Nevertheless with the LC-MS/MS method a higher PA concentration was obtained than with the traditional method. This can only be explained by the fact that losses occur during zinc reduction and/or in the liquid–liquid purification step of the traditional method. Therefore it is preferable to use the novel LC-MS/MS method; without reduction and purification to minimise PA loss.

For a high sample throughput it is desirable to minimise the complexity of the extraction procedure and to minimise the number of analytical steps to obtain the final extract. The extraction method of the novel technique, namely with formic acid without any extra steps (except a simple dilution step), is far less complex and time-consuming compared with the traditional method: extraction with sulfuric acid, reduction with zinc and purification for GC-NPD analysis.

The traditional method for PA analysis by GC-NPD and the newer method for PA analysis by LC-MS/MS were compared by measuring a number of dried *J. vulgaris* samples. The latter technique performed better with respect to sensitivity, simplicity and selectivity. Analysis by LC-MS/MS tolerates a much simpler sample treatment procedure (formic acid extraction, without the requirement of reduction and acid/base extraction steps) than the classical method with GC analysis. Reduction of analytical steps will in the end result in a higher sample throughput and this will allow more comprehensive studies on PA analysis of plant material. The simultaneous detection of PA *N*-oxides and tertiary amines in extracts widens the possibility of investigation of these two forms in biological matrices (plants as well as

invertebrates and mammals). LC-MS/MS intrinsically offers a much higher sensitivity than analysis by GC. It is not necessary to use this higher sensitivity to the full in the case of plant extracts where there is a plentiful supply, but it will be very valuable when minute amounts of material have to be analysed or when trace levels in biological samples need to be determined. This study shows that the concentrations measured in plant material by LC-MS/MS are higher than those measured by GC-NPD. This strongly indicates that losses occur during the reduction and purification steps required for GC-NPD analysis. These losses are minimised in the LC-MS/MS method.

Based on the simple and rapid sample preparation, sensitivity and discrimination between the two PA forms, formic acid extraction in combination with LC-MS/MS is the method of choice for determining PAs in plant material.

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