Pyrrolizidine Alkaloids in Honey: Comparison of analytical methods

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Abbreviations:

GC-EI-MS (SIM), gas chromatography electron ionization mass spectrometry operated in the selected ion monitoring mode; GC-MS, gas chromatography-mass spectrometry; HRGC-EI-MS (SIM), high resolution gas chromatography electron ionization mass spectrometry operated in the selected ion monitoring mode; HRGC-MS, high resolution gas chromatography-mass spectrometry; HPLC-MS, high-performance liquid chromatography-mass spectrometry; HPLC-MS-MS, high-performance liquid chromatography-tandem mass spectrometry; LC-ESI-MS, liquid chromatography electrospray ionization mass spectrometry; LC-MS, liquid chromatography-mass spectrometry; LC-MS-MS, liquid chromatography-tandem mass spectrometry; LLE, continuous liquid-liquid extraction; LOQ, limit of quantification; MSTFA, *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide; PA, pyrrolizidine alkaloid; PANOs, pyrrolizidine alkaloid *N*-oxides; RSD, relative standard deviation; SD, standard deviation; S/N, signal-to-noise ratio; SPE, solid phase extraction; SCX, strong cation exchange; SIM, selected ion monitoring; TMS, trimethylsilyl group.

Introduction

Pyrrolizidine alkaloids (PAs) are a group of constitutively expressed toxic plant defense compounds found in an estimate of 6000 flowering plants world wide (Stegelmeier et al. 1999; Roeder 2000). These plants generally belong to one of four plant families within the angiopserms: the Asteraceae (tribes Senecioneae and Eupatorieae), the Boraginaceae, the Apocynaceae and the genus *Crotalaria* within the Fabaceae (Hartmann and Witte 1995).

In the past decades a multitude of biological cross connections involving PAs were revealed. The effects and/or impacts of PAs range from deterrence, acute toxicity and genotoxicity to adaptation, dependence or profitable use by specialized herbivores and on to utilization of PAs in propagation and/or pheromone chemistry (Hartmann and Witte 1995). The structural diversity of 400+ known PAs and the broad range of polarity typically caused by the co-existence of tertiary PAs and PA-*N*-oxides (PANOs) represent a particular analytical challenge. As a consequence, a large variety of different extraction and concentration procedures, as well as analytical methods were developed over the years (for review see, Mattocks 1986; Rizk 1991). An update, taking into account the increasing importance of high-performance liquid chromatography-mass spectrometry (HPLC–MS) techniques, was published recently (Crews et al. 2010).

However, returning to honey, PAs were frequently detected throughout the last 30 years (for review see, Kempf et al. 2010a). Earlier studies were focused more or less on certain honeys derived from known or suspected PA-plants. Hence, only a clearly pre-defined set of PAs that needed to be considered. Recently, two studies were conducted on retail honey with unknown history and/or origin (VWA 2007; Kempf et al. 2008). Both studies pursued individual approaches and methodologies to measure low ppb-levels of PA in honey. While the first method represents a target HPLC-MS-MS approach and covered 11 PAs and PANOs reference compounds, the second method is a sum parameter method,

that uses GC-MS to detect the common backbone of most toxic relevant 1,2-unsaturated PAs.

So far, no commonly accepted method exists on how to analyze PA in honey. In cooperation with Intertek Food Services GmbH (a laboratory specialized in food analysis) a LC-ESI-MS-MS (liquid chromatography electrospray ionization mass spectrometry) based method was established which was intended to be applied to analyze honey from all over the world. In the interest of a better estimation of the potential of LC-MS-MS *vs*. GC-MS sum parameter three sample sets of honey were analyzed with both methods.

The results for different sample sets are discussed with respect to pros and cons for both methods.

Material and Methods

Chemicals and solvents

All chemicals were of analytical reagent purity and purchased from Acros Organics (Geel, Belgium), Fluka (Buchs, Switzerland), Merck (Darmstadt, Germany), Sigma-Aldrich (Steinheim, Germany) and Roth (Karlsruhe, Germany). All solvents were of HPLC grade purity or redistilled before use.

Reference materials

For the determination of PAs in honey by LC-MS analysis the reference materials echimidine, heliotrine, lasiocarpine, lycopsamine, monocrotaline, retrorsine, senecionine, seneciphylline and senkirkine (Figure 1) were obtained from Phytolab GmbH & Co. KG, Germany. Isoproturone-d6 (internal standard) was obtained from Dr. Ehrenstorfer GmbH, Germany. The *N*-oxides of echimidine, heliotrine, lycopsamine, monocrotaline, retrorsine, senecionine and seneciphylline were chemically synthesized and purified by a standard method (Cymerman Craig and Purushothaman 1970). Stock solutions (10 mg/L) were prepared in methanol.

To evaluate the GC-MS sum parameter method six PAs (Figure 1), monocrotaline (Sigma-Aldrich, Steinheim, Germany), senecionine (Roth, Karlsruhe, Germany), seneciphylline (Roth, Karlsruhe, Germany), senkirkine (Roth, Karlsruhe, Germany), heliotrine (Latoxan, Valence, France) and retrorsine (Sigma-Aldrich, Steinheim, Germany) were purchased. PAs and PANOs mixtures from *S. vernalis* were applied as previously described in Kempf et al. (2008).

GC-MS sum parameter method

The applied method was described in detail previously (Kempf et al. 2008). In all samples, except sample set C, 20 g of honey were used per work-up. Due to the high PA-contents

measured in sample set C, the sample weight was reduced for these samples to 5 g of honey per work-up, to fit into the calibrated range. The sequence of individual steps is summarized in a flow diagram (Figure 2).

GC-MS was carried out with a Fisons Instruments GC 8060 (Thermo Electron, Dreieich, Germany) gas chromatograph with split/splitless injection (220°C / 1:20) directly coupled to a Fisons Instruments MD 800 mass spectrometer (Thermo Electron, Dreieich, Germany) essentially as described in Kempf et al. 2008.

Standard controlled relative quantification with heliotridine (originated from 2 µg heliotrine per sample) as internal standard was performed by HRGC-MS under the above mentioned conditions. Linear retention indices were 1600 and 1632 for di-TMS-retronecine and the standard di-TMS-heliotridine, respectively. Integration of peak area counts in EI-MS SIM-mode (*m/z* 93, 183 and 299) was carried out. The relative intensities of these analytical ions to each other were used as tool for the determination of the purity of the corresponding peak. These values were compared to values obtained from authentic reference compounds; variances of < 10% were tolerated. No extraction/response factors (F = 1.0) were considered. The data obtained was finally calculated into retronecine equivalents as previously described in Kempf et al. 2008.

LC-ESI-MS-MS method

Samples were analyzed using a Thermo Fisher Surveyor autosampler and liquid chromatography system coupled to a triple quadrupole mass spectrometer TSQ Quantum Ultra (ThermoFisher, San Jose, CA, USA). Samples were injected onto a 150 x 2.1 mm, 5 µm Thermo Hypersil Gold reversed-phase column (ThermoFisher, San Jose, CA, USA). The sample preparation was realized by liquid-liquid extraction followed by online solid-phase extraction (online SPE) for cleanup. For on-line SPE clean-up the SPE2000 module (Intertek Food Sevices, Bremen, Germany) was used. The module includes a pump and

two 6-port swichting valves. The PAs are trapped on an on-line SPE-cartridge (10 x 4 mm, 5 µm Thermo Hypersil Gold ,ThermoFisher, San Jose, CA, USA). Subsequently, the column is washed with water to rinse and minimize matrix compounds. The analytes are eluted in back-flush direction to the LC-MS for detection. The cartridge is reconditioned for the next sample afterwards. The detailed programming of the SPE2000 module is added to the supplement.

The PAs were eluted from the column with a gradient flow (0.2 mL/min) of 0.1% glacial acetic acid in water (mobile phase A) and 0.1% glacial acetic acid in methanol (mobile phase B). The gradient was held at 90% mobile phase A for 2 min before being ramped down to 10% over 8 min. This condition for elution was held for 5 min. The gradient was set back to start conditions and re-equilibrated for 5 min in preparation for the next sample injection.

The sample preparation is based on the QuEChERS method for determination of pesticides in food matrices (Anastassiades et al. 2003). All honey samples were spiked with the internal standard isoproturone-d6 (100 µg kg^{-1}) before sample preparation. The honey samples (1 g) were diluted with water (4 mL) and homogenized for 5 min. 1.3 g QuEChERS-salt (0.8 g $MgSO_4$; 0.2 g NaCl; 0.1 g citric acid disodium salt sesquihydrate; 0.2 g sodium citrate dihydrate) and 4 mL acetonitrile were added, extracted for 15 min and centrifuged at 10000g for 6 min. 3.5 mL of the upper phase was transferred to another 12-mL centrifugation tube and 0.5 g $MgSO_4$ was added. After homogenization for 5 min and centrifugation at 10000g for 6 min 3 mL of the supernatant was transferred to another tube and evaporated to dryness. The residue was reconstituted with methanol/water solution (1 mL) containing 0.08% glacial acetic acid in water (900 µL) and methanol (100 µL). 100 µL were analyzed using LC-online-SPE-MS. For the cleanup step by online-SPE a 10 x 4 mm, 5 µm Thermo Hypersil Gold (ThermoFisher, San Jose, CA, USA) column was used. An overview of the individual steps in given in Figure 2.

Mass spectrometric data were collected in the positive heated-ESI-mode (electrospray ionization). The capillary temperature was 300°C at a spray voltage of 5.8 kV. The sheath gas flow was 25 arb units and the aux gas 5 arb units, respectively. The protonated molecules $[M+H]^+$ of PAs and corresponding *N*-oxides were chosen as the parent ions for fragmentation (for more details see supplement). At least 2 product ions of every PA were determined for identification in accordance with the European commission decision 2002/657/EC (European Commission 2002). For quantification the samples were calibrated against matrix-matched standards (polyfloral honey). Extraction losses were corrected by an internal standard isoproturone-d6. Due to the lack of certified stable-isotope labeled PA-analogues this is an first approach for quality control. For validation a blank polyfloral honey was fortified with PAs and the internal standard. This was done in 6-fold at three levels (1.0 µg kg^{-1}, 1.5 µg kg^{-1} and 2.0 µg kg^{-1}) on three different days. Together with control samples (0 µg kg^{-1}), the fortified samples were processed using the described method. To investigate the influence of different honey-matrices on the PA determination, three typical honeys (acacia, polyfloral, honeydew) were analyzed (blank and fortified with 1 µg kg^{-1}). No matrix interference was observed at the specific retention time of the analytes for all tested matrices. Moreover matrix-matched standards at 0.5 µg kg^{-1}, 1 µg kg^{-1}, 1.5 µg kg^{-1}, 2.0 µg kg^{-1} and 4.0 µg kg^{-1} (n=6) were analyzed to verify the linearity of response versus concentration. Recoveries and RSD (relative standard deviation) were calculated at 1.0 µg kg^{-1}, using the average of the 1.0 µg kg^{-1} matrix-matched standard (n=6). The limit of quantification (S/N 10:1) of 1 µg kg^{-1} (1 ppb) can be applied for all PAs except monocrotaline and monocrotaline-*N*-oxide (50 ppb). Recoveries range from 97.5% (echimidine-*N*-oxide) to 104.6% (lycopsamine-*N*-oxide) and the RSD from 4.9% (heliotrine-*N*-oxide) to 38.8% (seneciphylline).

Furthermore the developed LC-MS method was successfully tested on matrices like bee pollen, milk products, PA-plants and feed. The sample preparation leads to clean sample

extracts and therefore to reproducible results at low PA-levels (1-10 µg kg^{-1}).

Retail honey samples

Pure *Echium* honeys (n=8), purchased via internet stores, were harvested in New Zealand in season 2006 (sample set A). Sample set B consisted of a set of random raw honeys (n=8) which were imported to Germany in 2008.

Honey from J. vulgaris (sample set C)

2007

To harvest honey on a location with high abundance of *J. vulgaris* colonies were prepared and placed in two locations in the Netherlands, Planken Wambuis (close to Ede; PW2007) and Sinderhoeve (Heelsum; SH2007), on June 13th, 2007. The colonies consisted of a one storey hive with ten frames, of which seven to eight with brood, and with about 12-15 thousand bees. The two side frames consisted of empty, fresh foundation comb. The colonies were provided with sugar dough on top of the hive.

At the location Sinderhoeve the field with *J. vulgaris* was mown on July 10th. On both locations honey was harvested on August 1st, 2007. At the location Sinderhoeve from both of the colonies a full frame with sealed honey was harvested. At Planken Wambuis one colony also produced a full frame with sealed honey, the other colony had only one frame with a little honey. At the moment of harvest around the location Sinderhoeve hardly any flowering *J. vulgaris* was left, some Asteraceae, white clover and some Brasicaceae flowered. Also the heather started to flower. On Planken Wambuis only very few flowering *J. vulgaris* plants were left. Other plants present in the area were thistle, some other Asteraceae including *Senecio inaequidens* and heather.

2008

Four locations in the Netherlands were set up: Groenendaal (south of Apeldoorn,

GR2008): Planken Wambuis, Oud Reemst (Otterloo, PW2008); Reijerskamp (Wolfheze, RK2008); Sinderhoeve, (SH2008). The colonies were similar to 2007, and were placed on July 2nd. On July 17th it was observed that most of the colonies did not produce much honey so far. On all locations in July honey bees have been observed visiting *J. vulgaris* flowers. The same was still true on July 28th, at that time most of the *J. vulgaris* had finished flowering, and the heather started flowering. Therefore on August 2nd the honey samples were harvested from the colonies: not one fully sealed comb was found, so sealed honey parts were cut from the frames as samples. About 110 to 350 mL of honey per colony were harvested by pressing and further purified by sieving. A second sample, after the honey flow of heather, was taken on October 2nd. From most of the colonies a full comb of honey could be harvested. Other available plants were *Prunella, Myosotis, Crepis, Matricaria, Cirsium, Achillea, Vicia, Trifolium, Cerastium, Hypericum, Epilobium* and *Viola.* After pressing the honey was sieved through a double honey sieve, and stored as separate lots in Greiner tubes of 50 ml in the freezer.

Pollen analysis

Method used at Intertek Food Services

Pollen analysis was performed according to the official German norm (DIN 2002) which is closely related to the IHC method (von der Ohe et al. 2004).

In this procedure 10 g of honey were mixed twice with 20 mL demineralized water and centrifuged at 1000g. After decanting the supernatant liquid the pellet was resuspended on microscopic slides. A droplet of glycerin/gelatin was added and closed by a cover glass. A total of 500 pollen grains was determined to the genus level, in some cases down to the species level, by microscopic observation at a magnification of 400x.

Method used in Wageningen (NL)

Sampling of pollen from honey and the determination of the botanical origin was performed

by Standard Operation Procedure 95 from bees@wur (P.O. Box 16, 6700 AA Wageningen Building 107, Droevendaalsesteeg 1, 6708 PB Wageningen The Netherlands). In this procedure 10 grams of honey mixed with 20 mL demineralized water is centrifuged, and the pellet is used to resuspend on microscopic slides, to which a droplet of glycerol/gelatin/fuchsin is added and which is closed by a cover glass. A total of 500 pollen grains was determined to the genus level, in some cases down to the species level, by microscopic observation at a magnification of 400x.

Results and Discussion

The GC-MS sum parameter method was already described in detail (Kempf et al. 2008; Kempf et al. 2010a; Kempf et al. 2010b). Here, only a brief overview and a flow chart (Figure 2) are given. All 1,2-unsaturated PAs of the retronecine-type (including the corresponding *N*-oxides) are converted into the common core structure retronecine which is subsequently converted into di-TMS-retronecine and analyzed by GC-EI-MS (SIM). Heliotrine is used as internal standard for quantification, resulting in di-TMS-heliotridine (a diastereomere of di-TMS-retronecine) and was separated from its diastereomere on a regular GC column. To reach a LOQ of 10 ppb (S/N of 7:1) the work-up required 20 g of honey, reduction of the *N*-oxides and pre-concentration by SPE on SCX material. A number of PA-plants are described as potential sources for PA-contamination of honey (Edgar et al. 2002). However this method does not require any information about the possible PA-plant sources beforehand to conduct the analysis and the result is expressed in retronecine equivalents.

To establish a new effective and problem adequate LC-MS method for the general PA analysis in honey we first had to identify the PA-structures that are most likely to appear in honey. To achive this, several hundred pollen analysis pollen results that were obtained by routine controls of raw honeys imported to Germany during the period 2007-2009 (at Intertek Food Services) were evaluated for the most frequent PA-plants. The result is summarized in Table 1. As second step, literature and in-house databases of PA-plants were analyzed to identify commercially available marker-PAs that reflect the relevant PA-plants (Table 1). During this study and to this day only a limited number of PA compounds was/is available (Figure 1) as certified standards while no commercial source for PANOs was available. The corresponding PANOs were obtained from the tertiary PA-references by standard chemical conversion (Cymerman Craig and Purushothaman 1970). For most of the known “trouble makers” in honey at least one marker PA could be assigned (Table

1), but it needs to be considered that it is in the nature of things that the determination of individual PAs with a rather limited set of reference compounds necessarily risks that not included PAs will be overlooked. However, this approach was tailored specifically to honey and should reliably indicate a PA-contamination in honey but quantitative analysis results can only be assigned to the target compounds.

Three distinct sample sets were analyzed with both methods. To compare both methods, it is necessary to consider the molecular weight of each PA and convert the results of the single PA-determination by LC-MS into retronecine equivalents to compare it to the result obtained from the GC-MS method (the original data, pollen analysis results etc. for all samples is supplied as supplemental data).

The first sample set (set A) was composed of monofloral honeys from New Zealand. The pollen analysis of all samples revealed 61% to 80% *Echium* pollen. Earlier studies demonstrated that honey from *Echium* spp. is dominated by the occurrence of echimidine (Beales et al. 2004). Figure 3 summarizes the results for both methods. The structural information of the initial PAs is lost in the GC-MS sum parameter method, but in this study we were able to retrace this information from the LC-MS approach. Therefore, the most prominent PA was echimidine (up to 99%) accompanied by small amounts (1% to 2%) of echimidine *N*-oxide and trace amounts of a lycopsamine-type PA (less than 0.2%). In addition, Figure 3 clearly demonstrates a good correlation for both methods for *Echium*-honeys, and suggests that the determination of echimidine/echimidine-*N*-oxide seems to reflect to a high degree the PA content of *Echium*-honey. Both, *E. plantagineum* (Patersons's curse) and *E. vulgare* (Viper's bugloss) are a very attractive nectar and pollen source for honey bees (Somerville 2005). Pollen analysis suggests *Echium*-occurrence in major honey exporting regions like Australia/New Zealand, South America and southern Europe (Kempf et al. 2008; Kempf et al. 2010a; Kempf et al. 2010b). Hence, it is absolutely essential to include echimidine/echimidine-*N*-oxide in a target analysis approach for the

determination of PAs in honey. Unfortunately however, the availability of echimidine is limited at the present time and the corresponding *N*-oxide was not available as certified reference material.

The second sample set (set B) consisted of eight randomly picked raw honeys which were imported to Germany in 2008 (details see supplement). The quantitative result for both methods is summarized in Figure 4. Again the obtained values (re-calculated in retronecine equivalents) are quite similar. It was striking, that also samples without any PAs, correlated for both methods, which proofed, that both methodologies worked reliable in a yes/no determination of possible PA-contamination of honey. This time the individual PA-pattern detected by the LC-MS method was characterized almost exclusively by lycopsamine-type PAs (one sample also contained 2% lycopsamine-type-*N*-oxide; and another sample had in addition to lycopsamine-type PAs 3% echimidine). In this context, lycopsamin-type-PA is the sum of all possible stereoisomers, including indicine, intermedine, lycopsamine, rinderine and echinatine. However, all five PAs are structurally closely related diastereomeres, a similar retention behavior and mass spectrometric response can be assumed and it seems reasonable to record them as sum of lycopsamine-type PAs without complete structural assignment. The positive result for sample B6 and B7 can be traced back to *Eupatorium* as floral source (see supplement). Hence, the detection of lycopsamine-type PA is the logical consequence. In contrast, for sample B2 only low amounts of *Echium* pollen were spotted (and are reflected in low echimidine concentration 15 µg kg^{-1}; retronecine equivalents) but at the same time high amounts of lycopsamine were found as well (607 µg kg^{-1}; retronecine equivalents). Given the present knowledge, lycopsamine is usually not dominating in *Echium* spp.. Thus, the result can be interpreted in three ways, either (i) pollen of a known PA-plant were overlooked; or (ii) pollen was not represented in the honey; or (iii) most likely, our list of forage plants is still incomplete but in this case the PA-pattern of the “unknown/not pollen

represented" plant is dominated by lycopsamine. Up to this day, only lycopsamine is available as reference compound at limited quantities and no commercial source for the corresponding *N*-oxide is available at the moment.

Special attention was given to samples (set C) from two consecutives seasons (2007 and 2008) which were obtained from consciously placed bee hives at sites with high abundance of *Jacobaea vulgaris* (*syn. Senecio jacobaea,* tansy ragwort) in the Netherlands. All four locations were located on rather poor sandy soils, in the Veluwe region. The Veluwe is a Natural Park Area in the Netherlands, in which several formerly agriculturally used fields are returned to nature conservation programs. Generally after a few years of succession such fields are dominated by tansy ragwort. Many of these sites consist of several tens of hectares. The sites Groenendaal and Planken Wambuis are in the center of the park, the Sinderhoeve and Reijerskamp closer to the edges.

This was considered a worst case scenario, since it was tried to force the bees to forage on tansy ragwort, which was the only abundant flowering species in these areas during July. To obtain a more realistic impression of possible contamination of honey harvested by bee keepers in this area, a second harvest from the same colonies was made at the beginning of October 2008. This honey included the heather honey flow from August typical for this region.

When honey was harvested in early August 2007 and 2008, it was found that the dominant pollen species in the honey were from the Clusiaceae *(Hypericum),* Fagaceae *(Castanea),* Brassicaceae *(Sinapis),* Fabaceae *(Trifloium, Lotus, Medicago)* and Ericaceae *(Erica and Calluna).* The honey harvested on October 6th, 2008 was dominated by the Ericaceae *(Calluna)*, and in one case Liliaceae.

Set C showed a large variation of the detected PA-content of the individual honeys for each method (see Figure 5). While the amount of PA-plant pollen was low in these honeys (0-6.3%) the total PA-amount in some cases reached extreme values (range: 0-13019 µg

kg^{-1}; average 1261 or 76 µ kg^{-1} for GC-MS and LC-MS, respectively). In average theses ragwort honeys showed PA levels that were 23 times higher than those found for retail honeys and showed a contamination rate of 86% (compared to 9% contamination rate recently found for retail honeys, Kempf et al. 2008). The small amount of ragwort pollen found in those samples also suggests that pollen cannot be the only source of PA-contamination in *Senecio*-honey. In fact, nectar has to contribute to reach those high levels.

As a general rule, the total PA-amounts in the GC-MS sum parameter approach were much higher (ranging from a factor of approximately 2 to 124; average factor of 17). However, at second glance, the data also showed very important correlations. Namely, all the samples that do not contain any or very small amounts of PA (below the detection limit of the GC-MS approach) were the same in both approaches. This leads to assume, that there was no false positive detection with either method and all positive samples (considering the different detection limits for both methods) were also detected with both approaches. Still, the strong discrepancy of the PA-positive samples needs some explanation. For a number of reasons, we are quite confident that the values obtained with the sum parameter approach are really representing the total amount of 1,2-unsaturated PAs. First, as mentioned above PA-negative is negative in both approaches. Secondly, in all other sample sets before we used 20 g of honey for the work-up, for set C honeys, only 5 g were used because the PA-content was too high for the calibrated range. Hence, false positive results or matrix effects which might add to the total PA-amount should be rather decreased than increased in this set. Another argument in favor for the correctness of the sum parameter results comes from biosynthesis studies of several *Senecio* spp. There, the primary and dominant formation of senecionine-*N*-oxide was demonstrated in roots (Hartmann and Toppel 1987; Toppel et al. 1987). Subsequently, senecionine-*N*-oxide gets translocated via the phloem to the remaining plant organs (Hartmann et al. 1989). During

this process several successive and simple transformations of the primary senecionine-*N*-oxide structure take place and result in a species, organ and individual specific PA-bouquet (Hartmann and Dierich 1998). Several studies have demonstrated the existence of different chemotypes for *J. vulgaris* (syn. *S. jacobaea*, tansy ragwort) (Witte et al. 1992; Macel et al. 2004). Particularly the study from Witte et al. (1992) seems to be important since it reports the results of more than hundred individual analyzed *J. vulgaris* flower heads. There, in any case senecionine and seneciphylline were generally not dominating, usually they were detected in trace amounts or sometimes absent. Instead, the dominant PAs in flower heads, depending on the chemotype, were jacobine or erucifoline/acetylerucifoline. Thus, given the biosynthetic background, the sensitive LC-MS target analysis of senecionine and seneciphylline will most likely detect a PA-contamination of honey derived from tansy ragwort. But, it will necessarily fall short of the real value since major PAs were not available as certified standards and therefore not included in the target PA-list.

However, the LC-MS determination showed another distinct feature. While in the sample sets A and B, PANOs were of no importance, in sample set C, the *N*-oxides of senecionine and seneciphylline represented in average 50% (ranging from 0 to 89%) of the total PA-amount. The reasons remain entirely unknown, but it demonstrates that also PANOs need to be addressed, either through reduction and determination as tertiary PA (like in the GC-MS approach, see Figure 2) or by an individual detection as *N*-oxide in a LC-MS approach which in turn would require reliable sources for authentic reference material.

In addition, no correlation could be found between the percentage *J. vulgaris* pollen in the honey and the PA-content of the honey (see supplement). However, the duration of *S. vulgaris* exposure is clearly reflected in the 2007 samples. There, the SH2007 samples showed significant lower PA levels which can be explained by the mowing of the *S. vulgaris* fields three weeks before harvest.

Conclusions and Summary

The comparison of both methods for the determination of PAs in honey strongly correlated for two reasons. First, both methods showed PA-positive results for the same samples and in addition, considering the different detection limits for both methods, the reverse is also true for detecting PA-free samples. However, a closer look at the different sample sets (A-C) indicated that for honeys from plant sources where only limited reference compounds are available, a targeted approach can lead to a significant underestimation of the true PA-content (set C). The data presented here, shows the critical points of two different approaches for the PA-determination in honey. As we learned during this study, both methods have strong advantages and drawbacks that need to be considered. These are categorized in a short tabular form in Table 2.

Finally, the choice of an appropriate method depends on many factors (time, costs, validations, LOQs, man power, availability of reference compounds etc.) and it will still need vivid discussions and improvements for both approaches until reliable and efficient PA-determinations will be possible. Currently, and focused only on gain of information, both methods supplement each other in perfect way, since valuable information, that was necessarily lost in a sum parameter approach was complemented by the single PA-determination.

In recent years increasing concerns were raised about fields and road sides which turned yellow during summer due to the high abundance of tansy ragwort. By putting bee colonies in areas covered with tansy ragwort the worst case was tested, and the contents of PAs found were the highest reported in honeys so far and exceed the levels found in commercially available honeys in an extreme case by a factor of more than 100 (sample C1-1, 13019 µg kg^{-1} compared to 120 µg kg^{-1}; Kempf et al. 2008). Together with the results obtained for *Echium* honeys, it seems evident that substantial efforts have to be made to

prevent these honeys to enter the food chain. According to present knowledge, the most effective way to address the problem would be the careful selection of the locations for the bee hives (Reinhard et al. 2009). Since honey is traded globally the range of foraging plants will be hard to predict for all geographic regions and from season to season. In such circumstances, there seems to be no alternative to an analytical monitoring of honey.

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Table 1: Major PA-containing bee forage plants identified by routine pollen analysis of raw honeys imported to Germany during the period 2007-2009 (n= several hundred; Intertek Food Services). The listed PA-structures and citations are exemplified. Where possible, honey related citations and structures were selected preferentially.

plant family	genus / species	PA structures[1]	nectar[2] / pollen[3] abundance	reference[4] / geographic distribution[5]
Boraginaceae	*Alkanna spp.*	triangularine, dihydroxytriangularine, 7-and 9-angeloylretronecine,	yes / unknown	Roeder 1995; El-Shazly et al. 1998 unknown
	Anchusa off.	**lycopsamine, intermedine**[6] and 7-acetyllycopsamine/intermedine	yes / normal	Hartmann and Witte 1995; El-Shazly et al. 1998 rare Europe: common in dune areas
	Borago off.	**lycopsamine**, **intermedine**[6], 7-acetyllycopsamine/intermedine, amabiline and supinine	yes / low to normal	Roeder 1995; Langer and Franz 1997 Northern Europe (UK/Scottland), New Zealand France, Chile, Italy Europe: used for oil seed production
	Cynoglossum off.	**echinatine**[6], **rinderine**[6], heliosupine, 7-angeloylheliotridine, 7-angeloyl-9- (2-methylbutyryl) and heliotridine	yes / normal to high	El-Shazly et al. 1996; Froelich et al. 2007 Central and East Europe (Austria, Hungary, Slovakia)

	Echium spp. *E. plantagineum* *E. vulgare*	echiumine, **echimidine**, echivulgarine, 7-O-acetylvulgarine, acetylechimidine, vulgarine, echiuplatine, uplandicine, leptanthine, echimiplatine and 7-O-acetyl lycopsamine-type	yes / high	Colegate et al. 2005; Boppré et al. 2008 worldwide
	Heliotropium spp. *H. amplexicaule* *H. europaeum*	**lycopsamine, indicine[6], heliotrine, lasiocarpine**, acetylindicine, and europine	yes / unknown	Beales et al. 2004 South Europe (Greece), Australia
	Lithospermum spp	**lycopsamine,** lithosenine and 12-acetyllithosenine	yes / sporadically individual pollen	Roeder 1995; Wiedenfeld et al. 2003
	Myosotis[6]	myoscorpine, scorpioidine , 7-acetylscorpioidine**,** symphytine	yes / low to high	Hartmann and Witte 1995; Roeder 1995 worldwide
	Symphytum spp.	**lycopsamine, intermedine[6]**, 7-acetyl lycopsamine type, derivatives, symlandine, symviridine, myoscorpine, symphytine, **echimidine** , uplandicine	yes / moderate	Röder 1995;Feng et al. 2009 common Central and East Europe (Romania, Slovakia)
Compositae	*Eupatorium spp.* *Eupatorium cannabinum*	**echinatine[6], lycopsamine-type PA[6]**,	yes / rather high	Boppré et al. 2008 Europe (wetlands), Central and South America (Mexico, Guatemala, Cuba,

				El Salvador)
	Senecio spp. *Jacobaea vulgaris (S. jacobaea)* *S. vernalis*	**senecionine, seneciphylline, senkirkine** senecivernine, spartioidine, integerrimine, jacobine, ridelliine, jacozine, erucifoline, jacoline retrorsine, jaconine, usaramine, eruciflorine, acetylerucifoline	yes / low to high	Witte et al. 1992; Toppel et al. 1987 Asia (China, India)
Ranunculaceae[7]	*Caltha spp.*	**senecionine**	no / high	Hartmann and Witte 1995 North America, Europe (Hungary, Romania, Italy, France, UK)
Fabaceae	*Crotalaria*	**monocrotaline**[8]	unknown / unknown	Hartmann and Witte 1995

[1] representative structures (tertiary PAs only) for this genus (reference compounds used in this study are printed in bold)
[2] bee keeper information: plant is a known nectar source for honey bees
[3] bee keeper information: pollen abundance in honey for this forage plant
[4] selected references on PA-content for this genus
[5] based on results for routine pollen analysis of import honeys
[6] isomer of lycopsamine
[7] little or no data available
[8] so far never detected in honey

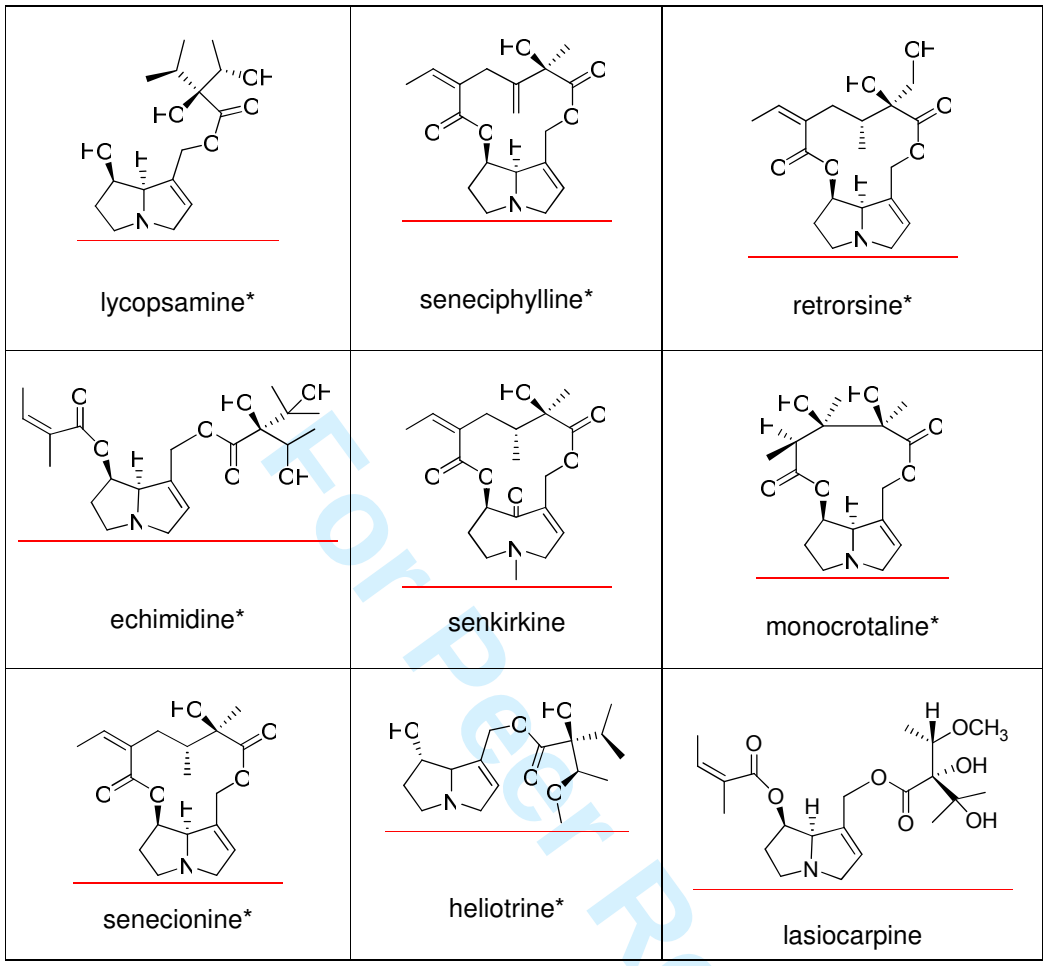

Figure 1: Structures of commercially available PAs used as reference compounds in this study. * The *N*-oxides of echimidine, heliotrine, lycopsamine, monocrotaline, retrorsine, senecionine and seneciphylline were chemically synthesized.

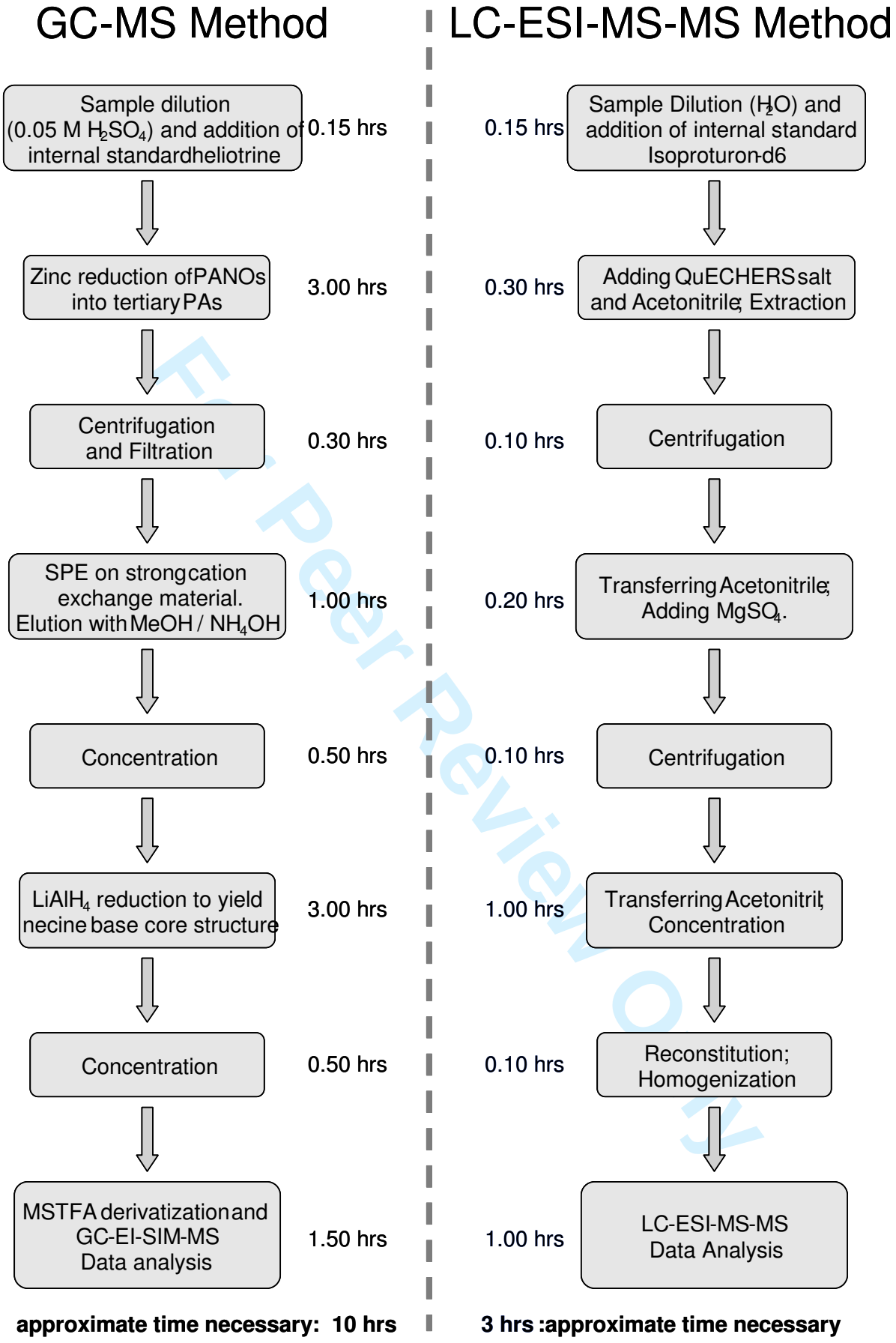

Figure 2: Schematic diagram of the key steps and the required time for sample preparation for both analytical methods.

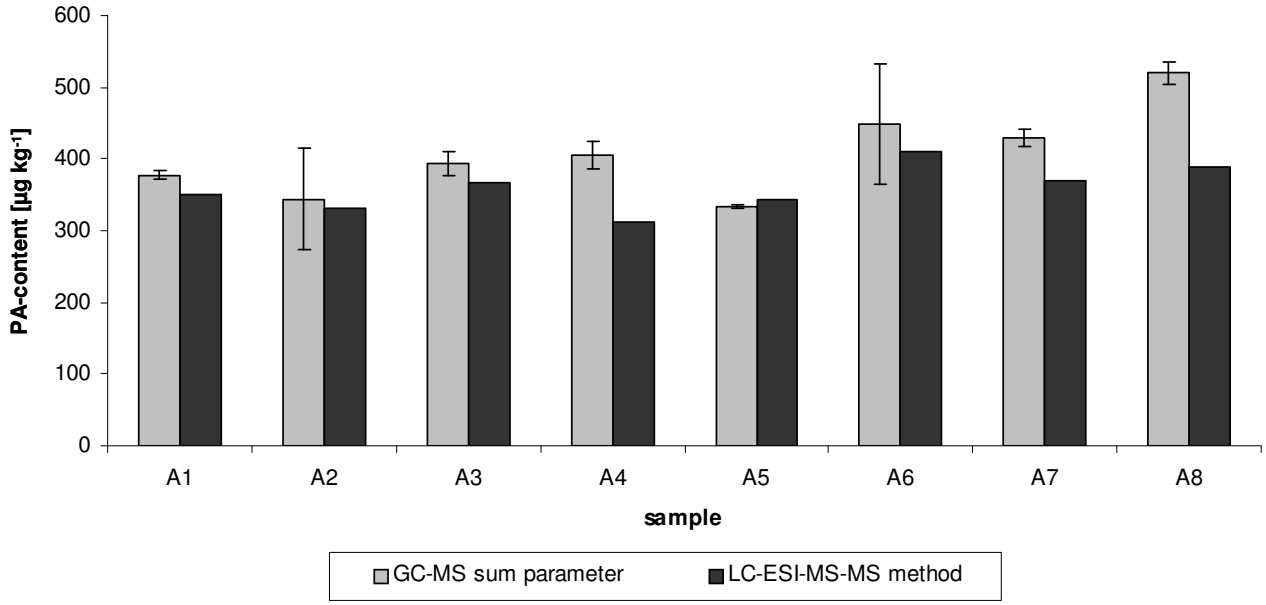

Figure 3: Comparison of the total PA-content of *Echium* honeys (n = 8; set A; New Zealand) calculated in retronecine equivalents. Left (gray) bars GC-MS sum parameter approach (SD; n = 3), right (black) bars LC-MS-MS method, respectively.

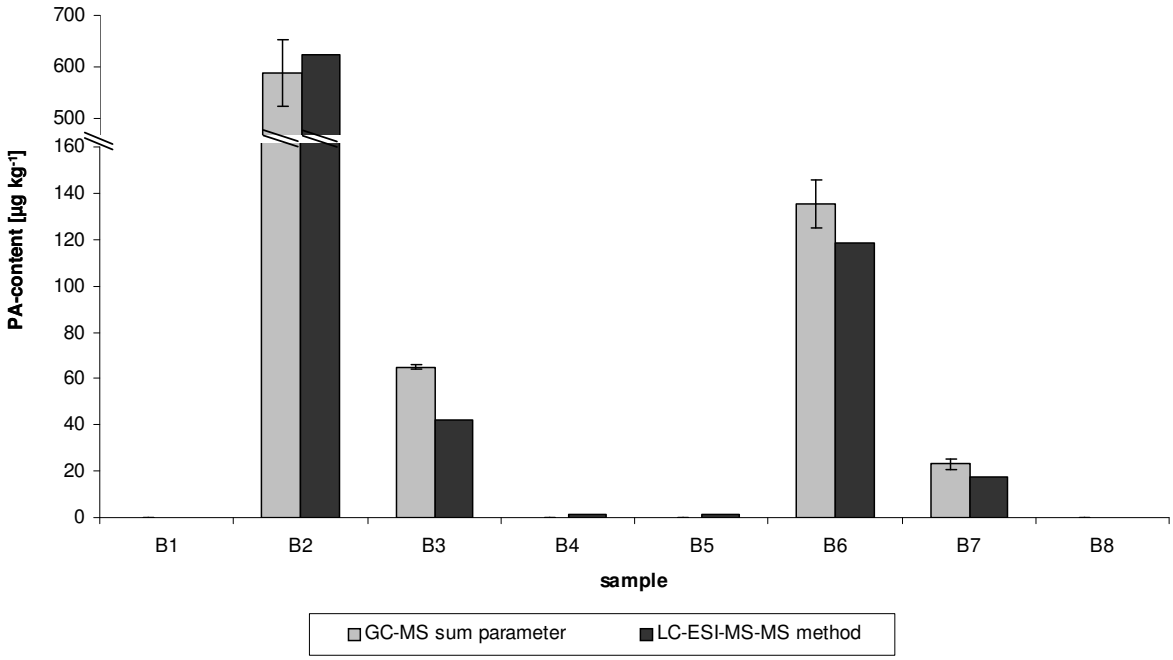

Figure 4: Comparison of the total PA-content of randomly picked raw honeys (n = 8; set B) calculated in retronecine equivalents. Left (gray) bars GC-MS sum parameter approach (SD; n = 3), right (black) bars LC-MS-MS method, respectively.

Table 2: Direct comparison of the advantages and disadvantages of both methods that were observed during the implementation of both methods

GC-MS sum parameter approach	**LC-ESI-MS-MS target PA approach**
Pros + low chance to miss toxic relevant PAs + true internal standard quantification + simple quantification + can be easily adapted to different samples and background + no need of background information of the samples or PAs involved + low dependency from standards + can be adapted to stable isotope dilution analysis	**Pros** + *N*-Oxides and tertiary PA can be determined simultaneously + proportion of each individual structure will be known which might be necessary for further toxicological aspects + low efforts for sample preparation + fast turnaround times + LOQ of 1 ppb + more informative (PA-plant patterns, ratio tertiary PAs/PANOs, link to geographic origin) + QuEChERS workup can be used
Cons - toxic otonecine-PAs are not covered - structural information of the original PAs is lost and possible differences between toxicological properties are not recorded - *N*-Oxide reduction is necessary - labor intensive sample work-up - double workup (with and without internal standard - lengthy turn-around time - LOQ of 10 ppb	**Cons** - unknown or unexpected PAs are missed - strong dependency on the availability of standard compounds - solid quantification needs many standard compounds - knowledge of the history of the samples is helpful - external quantification or quantification through standard addition - additional expenditure for data evaluation

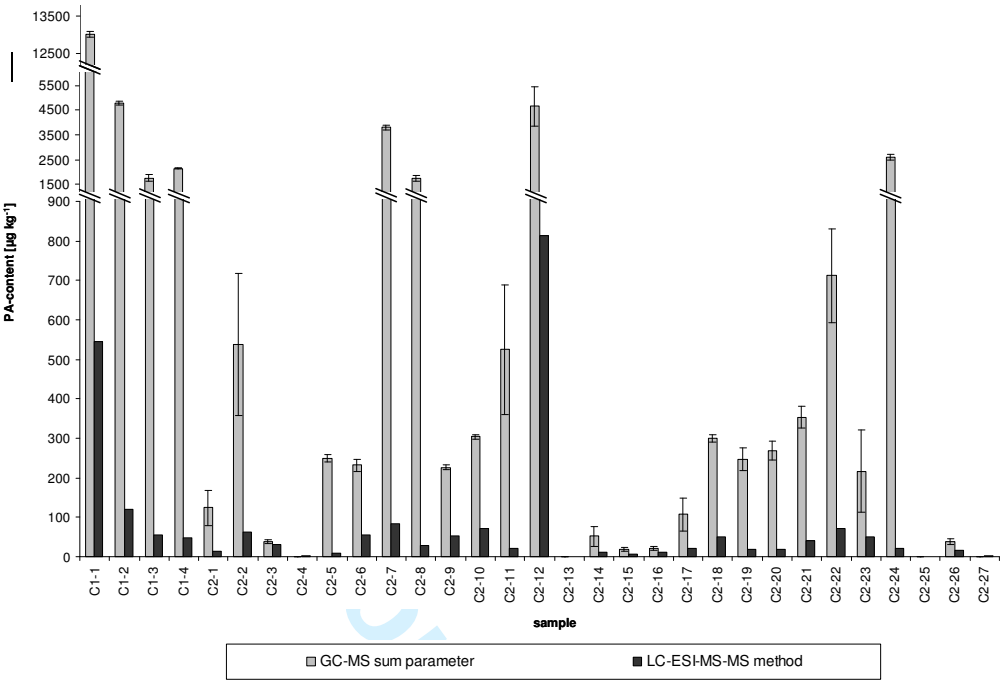

Figure 5: Comparison of the total PA-content of *Jacobaea vulgaris* honeys (n = 31; set C; Veluwe region; The Netherlands) calculated in retronecine equivalents. Left (gray) bars GC-MS sum parameter approach (SD; n = 3), right (black) bars LC-MS-MS method, respectively.

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Sample	Origin	PA-content [µg kg^{-1}]			Pollen analysis
		GC-MS sum paramter		LC-ESI-MS-MS	
		mean	SD (n=3)		
A1	New Zealand	378	6	351	61% *Echium* spp.
A2	New Zealand	344	71	332	62% *Echium* spp.
A3	New Zealand	394	17	367	62% *Echium* spp.
A4	New Zealand	406	20	311	68% *Echium* spp.
A5	New Zealand	334	3	344	66% *Echium* spp.
A6	New Zealand	449	84	411	61% *Echium* spp.
A7	New Zealand	429	12	370	72% *Echium* spp.
A8	New Zealand	520	15	389	80% *Echium* spp.

Sample	Origin	PA-content [µg kg^{-1}]			Pollen analysis
		GC-MS sum paramter		LC-ESI-MS-MS	
		mean	SD (n=3)		
B1	Cuba	traces	n.c.	0	9% *Eupatorium* spp.
B2	Australia	590	65	625	7% *Echium* spp.
B3	Cuba	65	1	42	sporadic *Eupatorium* spp.
B4	England	traces	n.c.	1	sporadic *Borago* spp.
B5	Spain	traces	n.c.	1	10% *Echium* spp.
B6	Cuba	135	10	119	7% *Eupatorium* spp.
B7	Guatemala	23	2	18	15% *Eupatorium* spp.
B8	Argentina	n.d.	n.d.	0	sporadic *Echium* spp.

n.d. = not detecable
n.c. = not able to calculate

Sample	Origin	PA-content [µg kg^{-1}] GC-MS sum paramter mean	SD (n=3)	LC-ESI-MS-MS	Pollen analysis
C1-1	the Netherlands, PW2007	13019	85	544	1,8% *Senecio* spp.
C1-2	the Netherlands, PW2007	4797	87	120	3,8% *Senecio* spp.
C1-3	the Netherlands, SH2007	1756	139	55	1,6% *Senecio* spp.
C1-4	the Netherlands, SH2007	2148	10	49	2,2% *Senecio* spp.
C2-1	the Netherlands, SH2008	124	45	15	1,6% *Senecio* spp.
C2-2	the Netherlands, SH2008	537	180	63	n.d.
C2-3	the Netherlands, SH2008	38	5	31	1,3% *Senecio* spp.
C2-4	the Netherlands, SH2008	n.d.	n.d.	3	n.p.
C2-5	the Netherlands, RK2008	250	10	10	0,6% *Senecio* spp.
C2-6	the Netherlands, RK2008	232	15	56	0,6% *Senecio* spp.
C2-7	the Netherlands, RK2008	3796	110	85	1,0% *Senecio* spp.
C2-8	the Netherlands, RK2008	1751	106	29	1,8% *Senecio* spp.
C2-9	the Netherlands, GR2008	226	6	52	n.p.
C2-10	the Netherlands, GR2008	304	6	73	n.p.
C2-11	the Netherlands, GR2008	525	165	22	n.p.
C2-12	the Netherlands, GR2008	4658	800	813	1,4% *Senecio* spp.
C2-13	the Netherlands, PW2008	n.d.	n.d.	0	n.p.
C2-14	the Netherlands, SH2008	52	25	11	1,0% *Senecio* spp.
C2-15	the Netherlands, SH2008	20	5	8	n.p.
C2-16	the Netherlands, SH2008	22	5	11	n.p.
C2-17	the Netherlands, SH2008	107	41	21	6,3% *Senecio* spp.
C2-18	the Netherlands, RK2008	300	10	51	n.p.
C2-19	the Netherlands, RK2008	248	29	20	n.p.
C2-20	the Netherlands, RK2008	268	24	19	n.p.
C2-21	the Netherlands, RK2008	354	28	42	n.p.
C2-22	the Netherlands, RK2008	712	118	73	n.p.
C2-23	the Netherlands, GR2008	217	104	51	n.p.
C2-24	the Netherlands, GR2008	2604	133	21	0,6% *Senecio* spp.
C2-25	the Netherlands, PW2008	n.d.	n.d.	0	n.p.
C2-26	the Netherlands, PW2008	38	7	16	n.p.
C2-27	the Netherlands, GR2008	n.d.	n.d.	2	n.p.

n.d. = no PA-plant Pollen detecable
n.p. = not performed

Single PA-content

sample	echimidine [µg kg^{-1}]	echimidine-*N*-oxide [µg kg^{-1}]	heliotrine [µg kg^{-1}]	heliotrine-*N*-oxide [µg kg^{-1}]	lycopsamine [µg kg^{-1}]	lycopsamin-*N*-oxide [µg kg^{-1}]	senecionine [µg kg^{-1}]	senecionin-*N*-oxide [µg kg^{-1}]	seneciphylline [µg kg^{-1}]	seneciphyllin-*N*-oxide [µg kg^{-1}]	senkirkine [µg kg^{-1}]	monocrotaline [µg kg^{-1}]	monocrotaline-*N*-oxide [µg kg^{-1}]	lasiocarpine [µg kg^{-1}]	retrorsine [µg kg^{-1}]	retrorsine-*N*-oxide [µg kg^{-1}]	total PA [µg kg^{-1}]
A1	885	11	0	0	2	0	0	0	0	0	0	0	0	0	0	0	898
A2	837	10	0	0	2	0	0	0	0	0	0	0	0	0	0	0	849
A3	929	9	0	0	1	0	0	0	0	0	0	0	0	0	0	0	939
A4	793	2	0	0	1	0	0	0	0	0	0	0	0	0	0	0	796
A5	875	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	880
A6	1046	3	0	0	2	0	0	0	0	0	0	0	0	0	0	0	1051
A7	944	3	0	0	1	0	0	0	0	0	0	0	0	0	0	0	948
A8	989	5	0	0	1	0	0	0	0	0	0	0	0	0	0	0	995
B1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B2	39	0	5	0	1171	0	0	0	0	0	0	0	0	0	0	0	1215
B3	0	0	0	0	80	0	0	1	0	0	0	0	0	0	0	0	81
B4	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	2
B5	2	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	3
B6	0	0	0	0	224	5	0	0	0	0	0	0	0	0	0	0	229
B7	0	0	0	0	34	0	0	0	0	0	0	0	0	0	0	0	34
B8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C1-1	0	0	0	0	0	0	513	80	579	4	0	0	0	0	0	0	1176
C1-2	0	0	0	0	0	0	115	22	95	30	0	0	0	0	0	0	262
C1-3	0	0	0	0	0	0	29	26	31	36	0	0	0	0	0	0	122
C1-4	0	0	0	0	0	0	29	18	29	32	0	0	0	0	0	0	108
C2-1	0	0	0	0	0	0	9	8	10	7	0	0	0	0	0	0	34
C2-2	0	0	0	0	0	0	13	49	17	62	0	0	0	0	0	0	141
C2-3	0	0	0	0	0	0	12	28	6	23	0	0	0	0	0	0	69
C2-4	0	0	0	0	0	0	3	0	3	0	0	0	0	0	0	0	6
C2-5	0	0	0	0	0	0	2	9	2	10	0	0	0	0	0	0	23
C2-6	0	0	0	0	0	0	27	39	26	32	0	0	0	0	0	0	124
C2-7	0	0	0	0	0	14	30	57	32	54	0	0	0	0	0	0	187
C2-8	0	0	0	0	0	0	3	24	4	34	0	0	0	0	0	0	65
C2-9	0	0	0	0	0	0	59	5	45	3	0	0	0	0	0	0	112
C2-10	0	0		0	0	0	30	57	23	52	0	0	0	0	0	0	162
C2-11	0	0	0	0	0	0	7	16	8	19	0	0	0	0	0	0	50
C2-12	0	0	0	0	0	0	419	364	440	570	0	0	0	0	0	0	1793
C2-13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C2-14	0	0	0	0	0	0	7	9	5	3	0	0	0	0	0	0	23
C2-15	0	0	0	0	0	0	6	6	4	1	0	0	0	0	0	0	17
C2-16	0	0	0	0	0	0	6	12	3	4	0	0	0	0	0	0	25
C2-17	0	0	0	0	0	0	11	17	8	10	0	0	0	0	0	0	46
C2-18	0	0	0	0	0	0	21	33	17	42	0	0	0	0	0	0	113
C2-19	0	0	0	0	0	0	21	8	12	3	0	0	0	0	0	0	44
C2-20	0	0	0	0	0	0	22	6	12	1	0	0	0	0	0	0	41
C2-21	0	0	0	0	0	0	11	28	14	41	0	0	0	0	0	0	94
C2-22	0	0	0	0	0	0	28	51	25	58	0	0	0	0	0	0	162
C2-23	0	0	0	0	0	0	34	35	29	14	0	0	0	0	0	0	112
C2-24	0	0	0	0	0	0	18	9	16	3	0	0	0	0	0	0	46
C2-25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C2-26	0	0	0	0	0	0	2	8	4	21	0	0	0	0	0	0	35
C2-27	0	0	0	0	0	0	2	0	2	0	0	0	0	0	0	0	4

Single PAs calculated as retronecine equivalents

sample	echimidine (MW 397.46) [µg kg^{-1}]	echimidine-*N*-oxide (MW 413.46) [µg kg^{-1}]	heliotrine MW 313.39 [µg kg^{-1}]	heliotrine-*N*-oxide (MW 329.39) [µg kg^{-1}]	lycopsamine (MW 299.36) [µg kg^{-1}]	lycopsamin-*N*-oxide (MW 315.36) [µg kg^{-1}]	senecionine (MW 335.39) [µg kg^{-1}]	senecionin-*N*-oxide (MW 351.39) [µg kg^{-1}]	seneciphylline MW 333.38) [µg kg^{-1}]	seneciphyllin-*N*-oxide (MW 349.38) [µg kg^{-1}]	senkirkine MW 365.39 [µg kg^{-1}]	monocrotaline (MW 325.36) [µg kg^{-1}]	monocrotaline-*N*-oxide (MW 341.36) [µg kg^{-1}]	lasiocarpine (MW 411.49) [µg kg^{-1}]	retrorsine MW 351.39 [µg kg^{-1}]	retrorsine-*N*-oxide (MW 367.39) [µg kg^{-1}]	total PA [µg kg^{-1}]
A1	346	4	0	0	1	0	0	0	0	0	0	0	0	0	0	0	351
A2	327	4	0	0	1	0	0	0	0	0	0	0	0	0	0	0	332
A3	363	3	0	0	1	0	0	0	0	0	0	0	0	0	0	0	367
A4	310	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	311
A5	342	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	344
A6	408	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	411
A7	369	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	370
A8	386	2	0	0	1	0	0	0	0	0	0	0	0	0	0	0	389
B1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B2	15	0	2	0	607	0	0	0	0	0	0	0	0	0	0	0	625
B3	0	0	0	0	42	0	0	0	0	0	0	0	0	0	0	0	42
B4	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1
B5	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1
B6	0	0	0	0	116	2	0	0	0	0	0	0	0	0	0	0	119
B7	0	0	0	0	18	0	0	0	0	0	0	0	0	0	0	0	18
B8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C1-1	0	0	0	0	0	0	237	35	270	2	0	0	0	0	0	0	544
C1-2	0	0	0	0	0	0	53	10	44	13	0	0	0	0	0	0	120
C1-3	0	0	0	0	0	0	13	11	14	16	0	0	0	0	0	0	55
C1-4	0	0	0	0	0	0	13	8	13	14	0	0	0	0	0	0	49
C2-1	0	0	0	0	0	0	4	4	5	3	0	0	0	0	0	0	15
C2-2	0	0	0	0	0	0	6	22	8	28	0	0	0	0	0	0	63
C2-3	0	0	0	0	0	0	6	12	3	10	0	0	0	0	0	0	31
C2-4	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	3
C2-5	0	0	0	0	0	0	1	4	1	4	0	0	0	0	0	0	10
C2-6	0	0	0	0	0	0	12	17	12	14	0	0	0	0	0	0	56
C2-7	0	0	0	0	0	7	14	25	15	24	0	0	0	0	0	0	85
C2-8	0	0	0	0	0	0	1	11	2	15	0	0	0	0	0	0	29
C2-9	0	0	0	0	0	0	27	2	21	1	0	0	0	0	0	0	52
C2-10	0	0	0	0	0	0	14	25	11	23	0	0	0	0	0	0	73
C2-11	0	0	0	0	0	0	3	7	4	8	0	0	0	0	0	0	22
C2-12	0	0	0	0	0	0	194	161	205	253	0	0	0	0	0	0	813
C2-13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C2-14	0	0	0	0	0	0	3	4	2	1	0	0	0	0	0	0	11
C2-15	0	0	0	0	0	0	3	3	2	0	0	0	0	0	0	0	8
C2-16	0	0	0	0	0	0	3	5	1	2	0	0	0	0	0	0	11
C2-17	0	0	0	0	0	0	5	8	4	4	0	0	0	0	0	0	21
C2-18	0	0	0	0	0	0	10	15	8	19	0	0	0	0	0	0	51
C2-19	0	0	0	0	0	0	10	4	6	1	0	0	0	0	0	0	20
C2-20	0	0	0	0	0	0	10	3	6	0	0	0	0	0	0	0	19
C2-21	0	0	0	0	0	0	5	12	7	18	0	0	0	0	0	0	42
C2-22	0	0	0	0	0	0	13	23	12	26	0	0	0	0	0	0	73
C2-23	0	0	0	0	0	0	16	15	13	6	0	0	0	0	0	0	51
C2-24	0	0	0	0	0	0	8	4	7	1	0	0	0	0	0	0	21
C2-25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C2-26	0	0	0	0	0	0	1	4	2	9	0	0	0	0	0	0	16
C2-27	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	2

LC-ESI-MS-MS transitions

pyrrolizidine alkaloid	parent-ion [m/z]	collision-energy	fragment-ions [m/z]	Rt [min]
Isoproturone-d6	213.0 $[M+H]^+$	18%	78+171	11.61
Monocrotaline	325.8 $[M+H]^+$	36%	120+121+94+194	4.99
Monocrotaline-*N*-oxide	341.8 $[M+H]^+$	38%	94+118+120+136+236	5.98
Echimidine	398.1 $[M+H]^+$	22%	120+220+336	8.40
Echimidine-*N*-oxide	414.1 $[M+H]^+$	30%	220+254+352+396	8.58
Heliotrine	314.1 $[M+H]^+$	25%	120+138.1+156	7.49
Heliotrine-*N*-oxide	330.1 $[M+H]^+$	30%	138+172+298	7.82
Lasiocarpine	412.1 $[M+H]^+$	30%	120+220+238+336+394	9.01
Lycopsamine	300.1 $[M+H]^+$	30%	94+120+138+156	6.42
Lycopsamine-*N*-oxide	316.1 $[M+H]^+$	35%	94+111+138+155+172	7.05
Retrorsine	352.0 $[M+H]^+$	36%	94+120+138+220	7.23
Retrorsine-*N*-oxide	368.0 $[M+H]^+$	37%	94 +120+136+138	7.50
Senecionine	336.1 $[M+H]^+$	35%	120+138+308	7.41
Senecionine-*N*-oxide	352.1 $[M+H]^+$	30%	118+120+136+220	7.75
Seneciphylline	334.1 $[M+H]^+$	35%	120+138+151+306	7.97
Seneciphylline-*N*-oxide	350.1 $[M+H]^+$	35%	118+120+136.1+322	8.32
Senkirkine	366.1 $[M+H]^+$	35%	122+150+168	8.54

Rt: retention time

SP2000 SPE-modul program

Step-Nr.	Time [min]	V1	V2	Pump (mL/min)
1	0,00	0	2	0,300
2	0,01	0	2	0,300
3	1,50	1	1	0,300
4	12,50	0	1	0,300
5	13,00	0	2	0,300
6	17,00	0	2	0,300

Valve 1 (V1): 0 Sample Application/ Cleanup/ Recondition
1 Elution

Valve 2 (V2): 1 Methanol + 1% CH_3COOH (v/v)
2 Water