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Senecio angustifolius as the major source of pyrrolizidine alkaloid contamination of rooibos tea (*Aspalathus linearis*)

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ABSTRACT

Pyrrolizidine alkaloids (PAs) and their *N*-oxides (PANOs) were detected in commercial rooibos tea which resulted in an investigation into the source of the contamination. Field studies showed that *Senecio angustifolius* occurs as a common weed throughout the production area and that it contains high levels of the same PAs (and in the same ratios) as those found in contaminated rooibos tea. The weed superficially resembles rooibos tea plants and is easily overlooked during weeding and harvesting. Analysis of a large number of plant material samples, collected from plantations from seven regions in the production area, showed that the rooibos plant (*Aspalathus linearis*) does not produce PAs. The detection of PAs in some rooibos plant materials from fields heavily infested with *Senecio angustifolia* can be explained by the recently demonstrated process of lateral transfer of PAs.

1. Introduction

Pyrrolizidine alkaloids (PAs) and their *N*-oxides (PANOs) are amongst the most widely distributed natural toxins produced by plants and are known to be hepatotoxic to humans and animals (Bull et al., 1968; Mattocks, 1986). They may cause acute liver damage (centrilobular hepatocellular necrosis) when ingested in large amounts but animal studies have also shown that some of them are genotoxic, carcinogenic and teratogenic, as indicated in reports by the German Federal Institute for Risk Assessment (Bundesinstitut für Risikobewertung or BfR) and European Food Safety Authority (EFSA) (EFSA, 2011; BfR, 2013a). More than 350 PAs have been described from 6000 plant species belonging mainly to the families Asteraceae (tribes Senecioneae and Eupatoriae), Boraginaceae and Fabaceae (Smith and Culvenor, 1981; Hartmann and Witte, 1995; EFSA, 2011; Langel et al., 2011).

There is currently a world-wide concern about the presence of PAs in food and beverage products such as honey (Kempf et al., 2008, 2010, 2011; Bodi et al., 2014; Martinello et al., 2014) and herbal teas (BfR, 2013a; Bodi et al., 2014; EMA, 2014; Mathon et al., 2014; Shimshoni et al., 2015), but also in medicinal products (EFSA, 2011; Codex Alimentarius Commission, 2014; Allgaier and Franz, 2015) and fodders (EFSA, 2011; Codex Alimentarius Commission, 2014). Trace amounts occur in many food items but the dietary relevance of such low levels in terms of health and safety is not yet fully known (BfR, 2013a, 2013b; EMA, 2014; Allgaier and Franz, 2015).

As part of a research project, the BfR first discovered the presence of PAs in rooibos tea [*Aspalathus linearis* (Burm.f.) R.Dahlgren, family Fabaceae] and in several other herbal teas such as fennel, chamomile, peppermint and nettle, as well as in green and black tea (BfR, 2013a). Other reports of herbal tea samples, including rooibos tea, confirmed that PA contamination is a general problem, not unique to rooibos tea. Samples collected for these reports came from retail markets in Switzerland (Mathon et al., 2014), Germany (Mädge et al., 2015), Belgium (Huybrechts and Callebaut, 2015) and Israel (Shimshoni et al., 2015). The alkaloid profile given for rooibos tea showed senecionine *N*-oxide as main compound (up to ca. 300 μg/kg), followed by lower levels of senecionine, retrorsine *N*-oxide, retrorsine, seneciphylline *N*-oxide, senecivernine *N*-oxide and a small amount of senkirkine (to yield total PAs of up to ca. 500 μg/kg) (Mathon et al., 2014). In an updated report on analyses of a wide range of teas and food items (Muller et al., 2015), the EFSA found the highest mean level of PAs in rooibos tea, namely 7.99 μg/L tea infusion.

Rooibos tea is very popular in South Africa, not only because it is produced here, but also for its caffeine-free status and beneficial health properties, in particular to relieve infantile colic. Major international markets are Germany, the Netherlands, UK, USA and Japan (Joubert and De Beer, 2011). The present study was initiated to address the

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urgent need to understand the various sources of PA contamination in rooibos tea and to identify the main source of contamination. It was also necessary to understand whether the rooibos plant produce PAs, given its close relationship with the genus *Crotalaria*, a well-known source of toxic PAs.

2. Materials and methods

A survey of the entire rooibos production area was conducted in September 2014 in the Western and Northern Cape Provinces of South Africa in seven regions, including two sites in the Clanwilliam area (listed from north to south – Fig. 1): (1) Nieuwoudtville, (2) Gifberg, (3) Agter-Pakhuis, (4a, 4b) Clanwilliam, (5) Paleisheuwel, (6) Citrusdal and (7) Piketberg. All potential sources of PA contamination, growing as weeds in and around rooibos tea plantations, were photographed, recorded and collected.

2.1. Samples

Samples of PA-producing weeds were collected in rooibos tea plantations (voucher specimen numbers in parenthesis, LV = Long and Van Wyk, all in JRAU): (1) *Amsinckia menziesii* (Lehm.) A. Nelson & J.F.

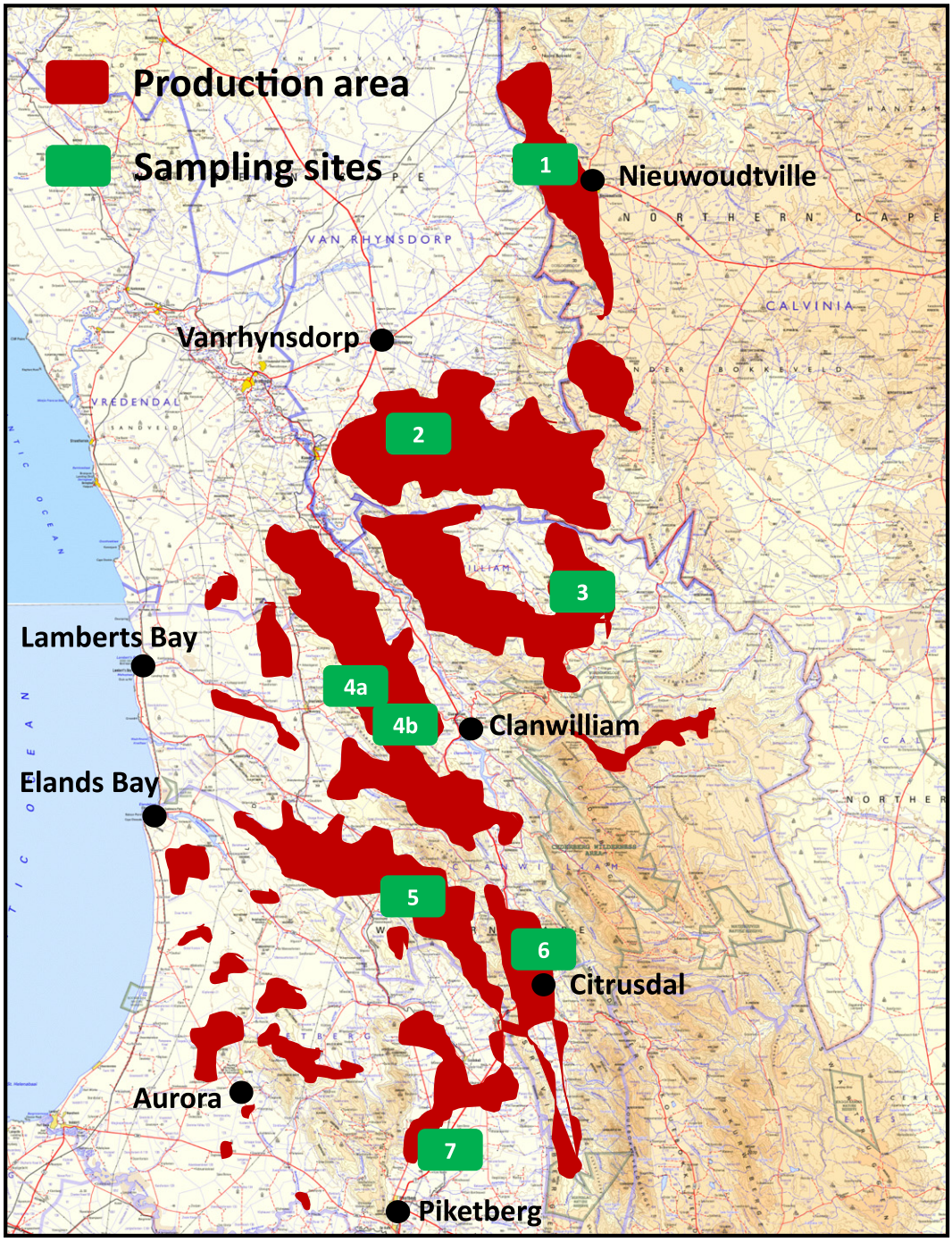

Fig. 1. Map of the rooibos tea production area in South Africa (in red) with sampling sites indicated in green.

Macbr. var. *intermedia* (Fisch. & C.A. Mey.) Ganders — common fiddleneck, from North America (LV56); (2) *Echium plantagineum* L. — Paterson's curse, from Europe, the Mediterranean region and Asia (LV44); (3) *Othonna coronopifolia* L. — *sandharpuis*, Cape endemic (LV58); (4) *Senecio angustifolius* (Thunb.) Willd. — *bitterbos* / "bitter bush", Cape endemic [Nieuwoudtville (LV30a,b,c); Gifberg (LV 32a,b,c); Agter-Pakhuis (LV 38a,b,c); Clanwilliam locality 1 (LV 31a,b,c); Clanwilliam locality 2 (LV 34a,b); Citrusdal (LV 35a,b,c); Paleisheuwel (LV 37a,b,c); Piketberg (LV 36a,b,c,d,e)]; (5) *Senecio arenarius* Thunb. — *hongerblom*, Namibia and Cape (LV 33a,b); (6) *Senecio burchellii* DC. — *geelgifbossie*, Namibia and Cape (LV 29 a,b). Fig. 2 depicts *Senecio angustifolia* plants within stands of rooibos tea (a and b), a flowering and fruiting plant (c) and a close-up of a flowering branch showing the terete leaves and discoid flower heads. Samples were air-dried at room temperature.

Samples of rooibos tea plants (*Aspalathus linearis*) were collected from commercial plantations at each of the seven regions (Fig. 1). Leafy twigs of six mature plants were collected at each locality, taking care to avoid any possible contact with other plants and weeds. These samples were thoroughly washed in cold water (to remove possible surface contaminants such as dust and pollen) before they were air-dried at room temperature in a separate laboratory to avoid any possible cross-contamination with the weed samples. Four commercial rooibos tea (product) samples that previously tested positive for PAs were also analysed for comparative purposes.

2.2. Extraction

Approximately 2 g of dry plant material of rooibos tea (commercial product or carefully air-dried leaves and stems) was ground in a pestle and mortar and extracted with 50% methanol in water acidified with 1% formic acid (15 ml). The plant material was soaked overnight in a 50 mL polypropylene centrifuge tube, followed by extraction in an ultrasonic bath (0.5 Hz, Integral systems, RSA) for 60 min at room temperature. The extracts were centrifuged (Hermle Z160m, 3000 ×*g* for 5 min) and the supernatants transferred to vials for analysis. The same procedure was followed for the *Senecio angustifolius* samples but these alkaloid-rich samples had to be analysed separately (see below).

2.3. Standards

Senecionine, senecionine *N*-oxide, senkirkine, retrorsine, retrorsine *N*-oxide and seneciphylline (PhytoLab GmbH & Co.KG, Vestenbergsgreuth, Germany) were used as external standards (Table 1) by preparing mixtures containing 0.2 μg/mL, 0.1 μg/mL, 0.02 μg/mL, 0.01 μg/mL and 0.002 μg/mL, respectively. The same solvent used for extraction of the samples was used to prepare the standards.

2.4. LC–MS analysis

All analyses were done at the Mass Spectrometry Unit of the Central Analytical Facility, University of Stellenbosch. The analytical method used was similar to that of Avula et al. (2015) but see also the methods proposed by the BfR (2013b, 2014). A Waters Synapt G2 Quadrupole time-of-flight (QTOF) mass spectrometer (MS) connected to a Waters Acquity ultra performance liquid chromatograph (UPLC) (Waters, Milford, MA, USA) was used for high resolution UPLC-MS analysis. Electrospray ionisation was used in the positive mode with the following operating parameters: cone voltage, 15 V; desolvation temperature, 275 °C; desolvation gas, 650 L/h. The remaining MS settings were optimised for best resolution and sensitivity. Data were acquired by scanning from *m/z* 200 to 1000 in resolution mode, as well as in MS^E mode. In MS^E mode two channels of MS data were acquired, one at a low collision energy (4 V) and the second using a collision energy ramp (40 to 100 V) to obtain fragmentation data. Leucine enkaphalin was used as lock mass (reference mass) for accurate mass determination and the instrument was calibrated with sodium formate. Separation was achieved on a Waters UPLC BEH C18 (2.1 × 100 mm; 1.7 μm particle size) column at 40 °C and a flow rate of 0.35 mL/min. An injection volume of 2 μl was used and the mobile phase consisted of 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B) in the following gradient: 95% A, 0–1 min; 95–90% A, 1–1.2 min; 90–60% A, 1.1–8 min; 60–20%, 8–10 min; 95% A, and 10–12 min.

Two rooibos tea samples (i.e. the dry processed plant material) that tested negative for PAs were spiked; the first sample to a final concentration of 4 μg/mL retrorsine and 4 μg/mL retrorsine *N*-oxide and the second sample to a final concentration of 4 μg/mL senecionine and

Fig. 2. *Senecio angustifolius*: a, b, Plants growing as a weed in plantations (indicated by arrows; S = *S. angustifolius*, R = *Aspalathus linearis*; note similarity between flowering plants of rooibos tea and those of *S. angustifolius* in b); c, flowering and fruiting plant; d, close-up of flowering branch showing the terete leaves and discoid flower heads. Photographs: B.–E. van Wyk.

Table 1
Distribution of the major pyrrolizidine alkaloids (as % of total alkaloids) in rooibos tea samples (1–4), rooibos plant samples (1–33) and *Senecio angustifolia* leaf samples (1–8). The rooibos plant samples and *S. angustifolia* samples are representative of the entire production area of rooibos tea (see Fig. 1). Settings for liquid chromatography–mass spectrometry (LC–MS/MS) analysis of the main alkaloids are also given.

LC–MS/MS settings and samples analysed	Presence of pyrrolizidine alkaloids (% of total)					
	Senecionine *N*-oxide	Retrorsine *N*-oxide	Retrorsine	Seneciphyline	Senecionine	Senkirkine
LC-MS/MS settings						
M + H	352.2	368.2	352.2	334.2	336.2	366.0
Product ion 1 (collision Energy, V)	118 (30)	154 (20)	120 (30)	120 (30)	120 (25)	150 (30)
Product ion 2 (collision Energy, V)	220 (25)	220 (30)	138 (30)	138 (30)	138 (30)	168 (28)
Cone Voltage (V)	30	30	30	20	15	30
Examples of contaminated rooibos tea samples						
Sample 1 (# 14037)	52.9	11.3	8.9	–	26.9	–
Sample 2 (# 14033)	63.8	13.3	7.2	–	14.7	1.0
Sample 3 (# 14018)	60.0	11.4	7.2	–	21.4	–
Sample 4 (# 14522)	59.8	17.6	5.5	–	16.0	1.1
Mean level	59.1	13.4	7.2	–	19.8	1.1
Rooibos plant samples, collected from the entire production area, September 2014 (1–24)						
1. Nieuwoudtville plant 1	–	–	–	–	–	–
2. Nieuwoudtville plant 2	–	–	–	–	–	–
3. Nieuwoudtville plant 3	–	–	–	–	–	–
4. Gifberg plant 1	–	–	–	–	–	–
5. Gifberg plant 2	–	–	–	–	–	–
6. Gifberg plant 3	–	–	–	–	–	–
7. Agter-Pakhuis plant 1	31.9	26.5	8.9	25.7	8	–
8. Agter-Pakhuis plant 2	–	–	–	–	–	–
9. Agter-Pakhuis plant 3	–	–	–	–	–	–
10. Agter-Pakhuis plant 4	–	–	–	–	–	–
11. Agter-Pakhuis plant 5	–	52.6	–	47.4	–	–
12. Agter-Pakhuis plant 6	62.7	–	–	–	37.3	–
13. Clanwilliam locality 1, plant 1	–	–	–	–	–	–
14. Clanwilliam locality 1, plant 2	–	–	–	–	–	–
15. Clanwilliam locality 1, plant 3	–	–	–	–	–	–
16. Clanwilliam locality 2, plant 1	–	–	–	–	–	–
17. Clanwilliam locality 2, plant 2	–	–	–	–	–	–
18. Clanwilliam locality 2, plant 3	–	–	–	–	–	–
19. Citrusdal plant 1	–	–	–	–	–	–
20. Citrusdal plant 2	–	–	–	–	–	–
21. Citrusdal plant 3	–	–	–	–	–	–
22. Paleisheuwel plant 1	–	–	–	–	–	–
23. Paleisheuwel plant 2	–	–	–	–	–	–
24. Paleisheuwel plant 3	–	–	–	–	–	–
25. Piketberg plant 1	57.8	11.1	–	11.1	20.0	–
26. Piketberg plant 2	100	–	–	–	–	–
27. Piketberg plant 3	91.4	–	–	–	8.6	–
28. Piketberg plant 4	78.6	–	–	–	21.4	–
29. Piketberg plant 5	60.4	–	15.8	–	23.8	–
30. Piketberg plant 6	80.7	4.8	–	3.4	23.8	–
Rooibos plant samples, collected at Paleisheuwel, February 2015 (25–33)						
25. Area 1 — no weeds nearby	–	–	–	–	–	–
26. Area 2 — no weeds nearby	–	–	–	–	–	–
27. Area 3 — no weeds nearby	–	–	–	–	–	–
28. Area 4 — no weeds nearby	–	–	–	–	–	–
29. Area 5 — no weeds nearby	–	–	–	–	–	–
30. Area 6 — no weeds nearby	–	–	–	–	–	–
31. Area 6 — *Senecio angustifolius* growing with the rooibos	37.7	27.4	28.3	–	11.1	–
32. Area 7 — no weeds nearby	–	–	–	–	–	–
33. Area 7 — *Senecio angustifolius* growing with the rooibos	43.7	32.2	13.9	–	10.2	–
Senecio angustifolia samples, collected from the entire production area, September 2014 (1–8)						
1. Nieuwoudtville	76.3	9.4	2.2	0.3	11.7	<0.1
2. Koebee	91.4	6.5	0.3	0.1	1.8	<0.1
3. Gifberg	76.4	13.8	2.5	0.2	7.2	–
4. Agter-Pakhuis	87.9	5.9	1.2	<0.1	4.4	0.6
5. Clanwilliam locality 1	71.2	22.3	3.1	0.2	3.2	–
6. Clanwilliam locality 2	69.5	18.4	3.3	1.0	7.9	<0.1
7. Citrusdal	67.8	11.2	4.3	0.5	16.2	–
8. Piketberg	68.9	26.6	1.4	0.2	2.9	<0.1
Mean level	76.2	14.3	2.3	0.3	6.9	0.1
Soil samples (1–9)						
1. Area 3 — soil collected at the roots of *Senecio pubigerus*	100	–	–	–		–
2. Area 7 — soil collected at the roots of *Senecio angustifolius*	80	–	–	–	20	–
3. Area 5 — soil collected from weed free area	–	–	–	–		–
4. Area 5 — soil collected at the roots of *Senecio angustifolius*	85	–	–	–	15	–

(continued on next page)

Table 1 (*continued*)

LC–MS/MS settings and samples analysed	Presence of pyrrolizidine alkaloids (% of total)					
	Senecionine *N*-oxide	Retrorsine *N*-oxide	Retrorsine	Seneciphyline	Senecionine	Senkirkine
5. Area 2 — soil from area with many weeds	–	–	–	–	–	–
6. Area 4 — soil from area with many weeds	–	–	–	–	–	–
7. Area 7 — soil from weed free area	100	–	–	–	–	–
8. Area 5 — soil from weed free area	–	–	–	–	–	–
9. Area 8 — soil from weed free area	–	–	–	–	–	–

4 μg/mL senecionine *N*-oxide. Similarly, contaminated samples were spiked to determine if suspected PA peaks increased in size upon spiking.

3. Results and discussion

3.1. Screening of rooibos plants for PAs

All rooibos plants collected from weed-free places tested negative (Table 1), confirming the apparent inability of the plant to synthesise PAs. It is noteworthy that the rooibos plants which tested positive for alkaloids came from plantations heavily infested with *Senecio angustifolius,* namely three of six samples from Agter-Pakhuis and all six samples from Piketberg (see discussion on lateral transfer below).

Aspalathus linearis belongs to the tribe Crotalarieae, a predominantly African group of some 1223 species (Van Wyk, 2005; Boatwright et al., 2008, 2011). Several genera and species of the tribe are known to produce quinolizidine and/or pyrrolizidine alkaloids. The relationships and patterns of alkaloid production amongst the genera of the Crotalarieae were therefore highly relevant in answering the important question: does *A. linearis* produce pyrrolizidine alkaloids?

The intricate relationships amongst the genera are well known, but chemosystematic (Van Wyk, 2003) and molecular systematic (Boatwright et al., 2008, 2011) studies have gone a long way towards resolving the phylogeny. Four main groups (clades) have been distinguished:

(1) Crotalaria group, comprising the genera *Crotalaria* (702 spp., many with high levels of PAs), *Bolusia* Benth. (7 spp., with some evidence of PAs) and *Euchlora* Eckl. & Zeyh. (1 sp., with PAs);
(2) Lotononis group, comprising the genera *Lotononis* (91 species, many with PAs, both saturated and unsaturated), *Leobordea* Delile (51 spp., some with trace amounts of quinolizidine alkaloids) and *Listia* E.Mey. (7 spp., with trace amounts of quinolizidine alkaloids);
(3) Pearsonia group, comprising *Pearsonia* Dümmer (13 spp.), *Rothia* Pers. (2 spp.) and *Robynsiophyton* R. Wilczek (1 sp.), all with high levels of various esters of quinolizidine alkaloids);
(4) Cape group, comprising *Ezoloba* B.-E.van Wyk & Boatwr. (1 sp., alkaloids unstudied), *Lebeckia* Thunb. (14 species, almost all with high levels of quinolizidine alkaloids and one with a PA base), *Calobota* Eckl. & Zeyh. (16 spp., some with high levels of quinolizidine alkaloids), *Rafnia* Thunb. (20 spp., quinolizidine alkaloids at best in trace amounts), *Wiborgiella* Boatwr.& B.-E.van Wyk (quinolizidine alkaloids at best in trace amounts), *Wiborgia* Thunb. (quinolizidine alkaloids at best in trace amounts) and *Aspalathus* L. (280 spp., a few with quinolizidine alkaloids but mostly trace amounts).

The most well-known source of toxic PAs in the Fabaceae is the large genus *Crotalaria* (702 species), which is a rich source of toxic PAs – amongst them also senecionine and retrorsine (Van Wyk and Verdoorn, 1990). *Euchlora* and some species of *Lotononis* produce small amounts of senecionine and/or integerrimine. Other groups typically have quinolizidine alkaloids, sometimes in quite high concentrations. The Cape group includes *Lebeckia* and *Calobota*, both accumulating high levels of quinolizidine alkaloids, but the rest of the group seems to be characterised by an almost complete loss of the ability to produce these alkaloids. Macrocyclic PAs have not yet been detected in any species of the Cape group. *Lebeckia wrightii* Bolus contains an as yet partially identified pyrrolizidine base (Van Wyk and Verdoorn, unpublished data). Several species of *Aspalathus* (including *A. linearis*) seem to produce trace amounts of quinolizidine alkaloids (mainly sparteine and lupanine) (Van Wyk and Verdoorn, 1989). *Aspalathus nivea* Thunb. is thus far the only species known to accumulate high levels of quinolizidine alkaloids (estimated at 2587 μg/g). *Aspalathus linearis* was reported to contain trace amounts of sparteine (Van Wyk and Verdoorn, 1990) but it will be worthwhile to reinvestigate the species of *Aspalathus* using sophisticated modern analytical methods. It should be noted that quinolizidine alkaloids are apparently harmless at very low concentrations, with no chronic effects (Wink and Van Wyk, 2008).

The reported presence of PAs such as retrorsine, senecionine and structurally related compounds (as reviewed above) was alarming, as it suggested that *A. linearis* may share with some of its relatives (*Crotalaria* and *Lotononis* species) the ability to accumulate PAs, albeit in low quantities. Even more alarming was the presence of PAs in leaves and twigs of *A. linearis* plants that were carefully sampled to avoid any possible contamination (Table 1, samples 1 to 33). The level of PAs in these samples varied from zero (mostly) to levels approaching or even exceeding the levels that were recorded in contaminated tea samples.

Further investigation revealed an explanation for the presence of PAs in rooibos plants, namely that it can be absorbed from the soil where rooibos plants co-occur with *Senecio* plants (Table 1, samples 31 and 33). Soil collected at the roots of *Senecio* species (Table 1, soil samples 1, 2 and 4) contained relatively high levels of senecionine and senecionine *N*-oxide. This preliminary study of carefully collected soil samples showed that soil can be contaminated with PAs and that this offers the only plausible explanation for the presence of PAs in rooibos tea plants. Carefully designed experiments are needed to confirm the lateral transfer of PAs from *Senecio angustifolius* to *Aspalathus linearis.* However, the lateral transfer of PAs from *Senecio jacobaea* L. to several other plant species was recently demonstrated by Nowak et al. (2016). In a series of experiments, these authors showed that all acceptor plants tested showed noteworthy levels of PAs and that the composition of imported PAs was specific to the acceptor plant species. These experiments proved beyond doubt that PAs are leached from dried *Senecio jacobaea* material into the soil and that the alkaloids are absorbed by the roots of other plants and translocated to the leaves (Nowak et al., 2016). Lateral transfer of PAs therefore also offers an explanation for the sometimes high levels of PAs detected in various other food and tea products where direct contamination seemed unlikely.

3.2. Main sources of PA contamination of rooibos tea

The field survey showed the presence of numerous weeds in plantations, often in relatively large quantities. For some years it has been common practice in the industry to produce rooibos tea in an ecologically friendly way and to avoid herbicides and pesticides. As a result, weed competition was not only tolerated, but considered beneficial to counteract the negative effects of monoculture. Most of the weedy PA-producing species such as *Amsinckia menziesii*, *Echium plantagineum*,

Othonna coronopifolia, *Senecio arenarius*, *S. burchellii* and *S. pubigerus* L. occurred only sporadically in one or a few plantations. Only *Senecio angustifolius* plants were found in abundance in practically all plantations visited (in the case of one exceptionally clean plantation, along the boundaries only). This weed is a perennial herb (shrublet) growing ca. 1 m (0.4–1.8 m) high, superficially similar to rooibos tea. It has thin erect stems, often reddish in colour, with linear to needle-shaped terete leaves and yellow discoid flower heads (i.e., lacking ray florets) of the same colour as rooibos flowers (Fig. 2c,d). In one well-weeded plantation, numerous *Senecio angustifolius* plants were growing (and flowering) *within* the rows of rooibos tea, obviously overlooked during the weeding operation (Fig. 2a,b). All other weeds were distinctly different from rooibos tea and therefore unlikely to be incorporated into the crop during tea harvesting. The closely related *S. burchellii* (*geelgifbossie*), which is also present on some farms, can easily be distinguished from *S. angustifolius* by the presence of ray florets.

Several vernacular names have been recorded for *S. angustifolius*. These are *bitterbos* ("bitter bush") in the Gifberg and Kobee areas — the plants are said to leave an intensely bitter taste on the hands when they were handled; *ghwanobos* ("ghwano bush") in the Agter-Pakhuis area — according to local farmers, "the plant only became a problem weed when we started to use fertilisers (ghwano)"; *sprinkaanbos* ("locust bush") — Ceres area; *dunsiektebos* ("thin sickness bush") in the Sutherland area — indicating that the plant causes chronic PA poisoning in livestock; and lastly *anysbos* ("anise bush") also in the Gifberg and Kobee area — it is said to superficially resemble wild anise (*Annesorhiza altiscapa* Schltr. ex H.Wolff) in appearance and smell. *Senecio angustifolius* has a localised distribution in South Africa that corresponds to the production area of rooibos tea (endemic to the Calvinia, Sutherland, Clanwilliam, Ceres, Worcester and Piketberg regions). The recent dominance of *Senecio angustifolius* can be attributed to: (1) Extension of the production area to shallower, heavier and more fertile soils. It is still rare or sporadic on infertile deep sands (but increases in high rainfall regions or where fertiliser is applied); (2) It is relatively inconspicuous and superficially resembles rooibos; (3) It is highly resistant to commonly used herbicides and is sometimes overlooked during weeding operations; (4) It germinates and grows throughout the year (unlike most other weeds, which are highly seasonal), and is therefore more difficult to control; and (5) The policy to minimise the use of chemicals and to allow weeds to grow as ground cover in plantations.

3.3. Analysis of PAs in Senecio angustifolius

The major PAs and PANOs were identified in the *Senecio angustifolius* samples collected for this study in the different localities based on their characteristic fragments at *m*/*z* 118, 120, 136 and 138, their accurate mass and relative retention times according to Avula et al. (2015) and Zhu et al. (2016). Chromatograms of two representative samples are shown in Fig. 3. It is evident that the main compounds present in these samples are senecionine *N*-oxide and retrorsine *N*-oxide, together with their respective isomers. The results for these compounds and their isomers were combined by integrating both peaks together (Table 1). A further 34 samples split into roots and leaves confirmed that senecionine *N*-oxide and retrorsine *N*-oxide are the main compounds, with average concentrations of respectively 76.2 and 14.3% of total alkaloids (Table 1), or respectively 83.1 ± 12% and 16.1 ± 12% when only the ratio of two main compounds are considered. Identification of the six major compounds was confirmed by injecting their commercial standards (Table 1). Most of the compounds had a peak with the same accurate mass and fragment ions eluting just before them (Fig. 3). Co-injection with the commercial standards showed only one of the peaks increased, indicating that it was not a solvent or chromatography related artefact. These were identified as the diastereoisomers, which was confirmed by GC–MS analysis (data not shown).

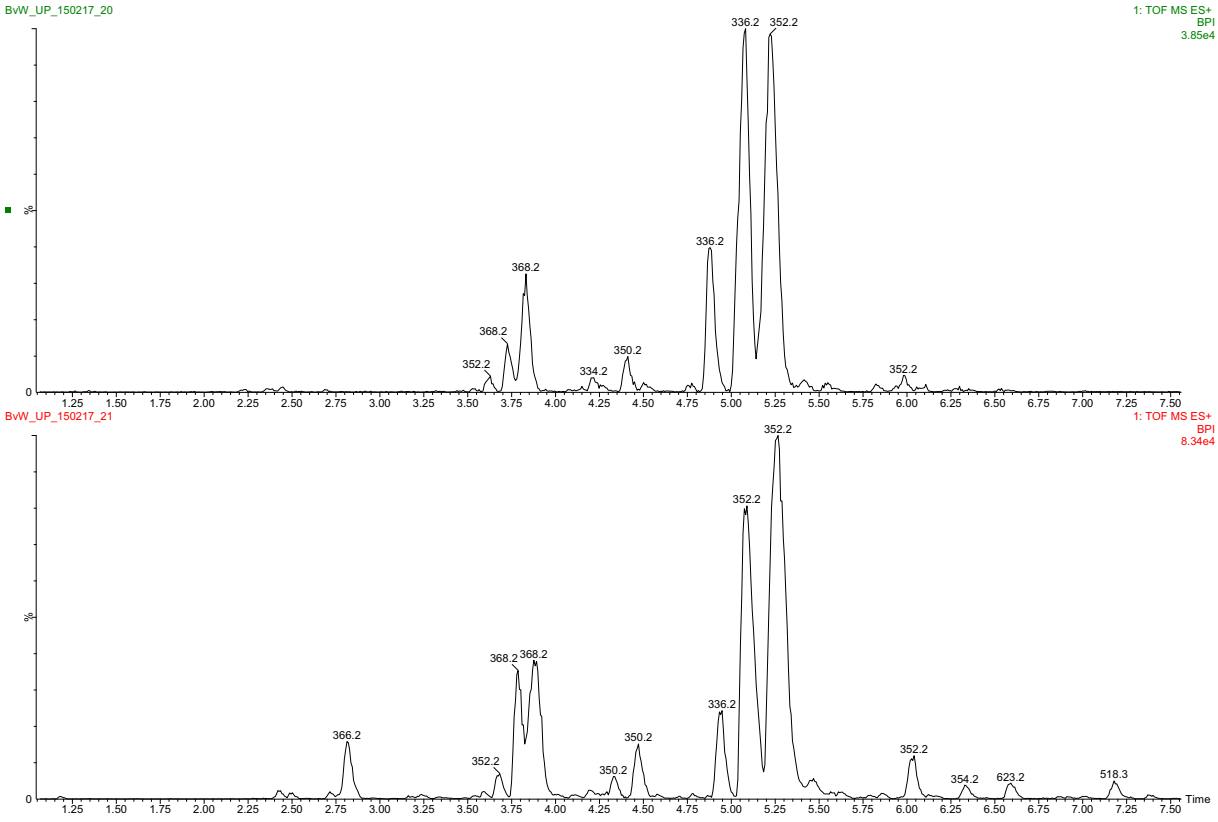

Fig. 3. Total ion chromatograms of two representative *Senecio angustifolius* extracts (Paleisheuwel, top and Gifberg, below). The main peak (retention time = 5.25 min; M + H = 352.2) represents senecionine *N*-oxide, while the peak at a retention time of 3.80 min (M + H = 368.2) represents retrorsine *N*-oxide.

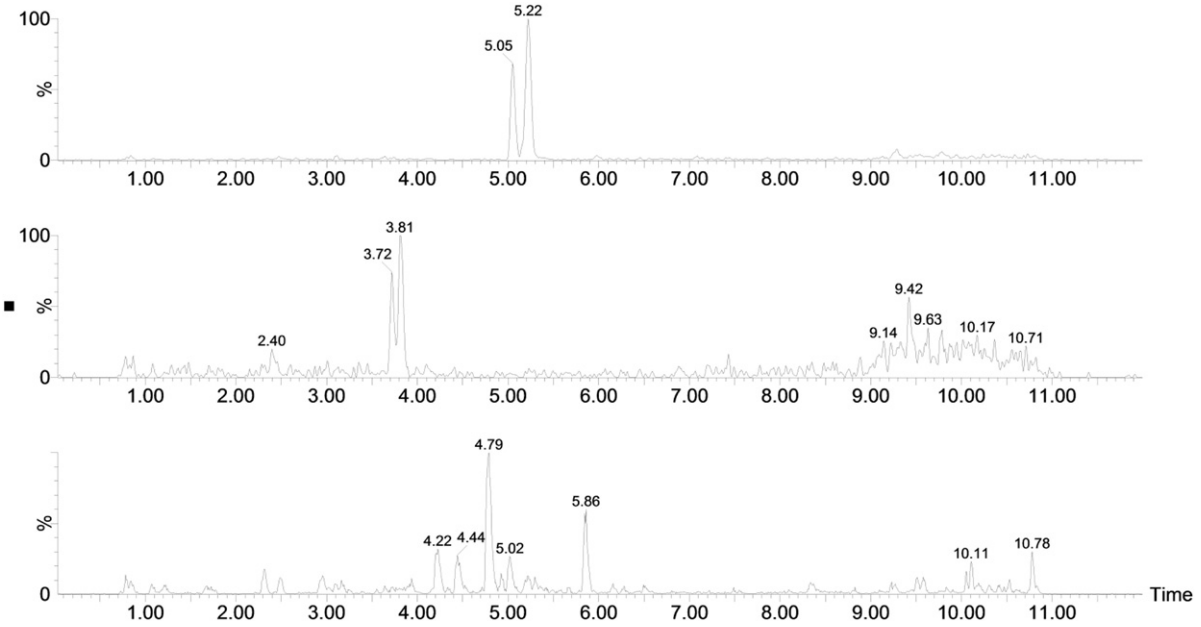

Fig. 4. Extracted mass chromatogram at *m/z* 352.2 (top) and 368.2 (middle) and total ion chromatogram (bottom) of a rooibos tea (*Aspalathus linearis*) extract that tested positive for pyrrolizidine alkaloids.

The expectation that commercial rooibos tea might have been tainted with *Senecio angustifolius* prompted us to use the same method to analyse a rooibos tea (product) sample that previously tested positive for PAs. It is clear from Fig. 4 that the same two PANOs, namely senecionine *N*-oxide (extracted mass chromatogram at *m/z* 352.2, retention time 5.22, top) and retrorsine *N*-oxide (extracted mass chromatogram at *m/z* 368.2, retention time 3.81, middle) and structurally related PAs identified in *S. angustifolius,* were also present in rooibos. A rooibos tea sample that tested negative for PAs (and other samples that have tested positive) was subsequently spiked with these two PANOs and their corresponding PAs, providing further confirmation of the identity of the main alkaloids. The results of the analyses of four typical PA-contaminated rooibos tea (product) samples are shown in Table 1. The mean concentrations of senecionine *N*-oxide and retrorsine *N*-oxide were respectively 59.1% and 13.4% of total alkaloids (Table 1), or respectively 81.5% and 18.5% when only the ratio of the two compounds are considered. These agree quite well with the corresponding percentages for *Senecio angustifolius* (71.5 ± 11% and 12 ± 7.8% of total alkaloids, in a ratio of 83.1 ± 12% and 16.1 ± 12%). The higher values for senecionine and retrorsine in the contaminated tea samples may indicate that the corresponding *N*-oxides are partially converted to their PAs during processing. Analyses of the closely related *Senecio burchellii* showed that although this species also accumulates senecionine *N*-oxide, it had higher levels of senkirkine (found only at very low levels in *S. angustifolius*). The sporadic occurrence of very small amounts of senkirkine in rooibos tea may therefore partly originate from *S. burchellii* (data not shown). The extremely high levels of PAs found in *S. angustifolius* means that even a relatively small quantity of the weed can result in detectable quantities of PAs in a large batch of rooibos tea.

The field studies revealed that *Senecio angustifolius* is the dominant pyrrolizidine-bearing weed throughout the production area of rooibos tea. What makes this plant particularly problematic is that it is easily overlooked during the harvesting and cutting of tea. As a result, it is also left behind in weeding operations and has undoubtedly been harvested with rooibos tea in the recent harvesting seasons, either accidentally or even deliberately (by unskilled labourers, who may have confused it with rooibos tea). The only long term solution appears to be the careful removal of all *Senecio angustifolius* plants (along with other pyrrolizidine-bearing species) while they are still conspicuous (growing and flowering). A careful inspection by skilled labour will be necessary prior to the actual harvesting operation to remove all plants that have previously been overlooked (especially those that grow intertwined with rooibos plants, young plants that may have subsequently germinated and mature plants on the edges and roadsides that may act as a source of windblown seeds). All workers need to be trained so that they would be able to easily identify *S. angustifolius* and other pyrrolizidine-bearing weeds. A recent code of practice (Codex Alimentarius Commission, 2014) contains valuable guidelines for the management of weeds to prevent and reduce PA contamination of food and feed. Further measures are also necessary to reduce or eliminate PA contamination of agricultural soils where food crops are grown.

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