

## Short communication

# Simultaneous quantification of anthrones and chromones in *Aloe ferox* (“Cape aloes”) using UHPLC–MS



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## ABSTRACT

*Aloe ferox* is an important medicinal plant indigenous to South Africa where it has primarily been used as a purgative and also to treat skin disorders. Modern day uses include; health (tonic) drinks, flavourant in alcoholic beverages, treatment of digestive disorders and inclusion in cosmetic formulations. Due to increased commercialization of aloe products, a rapid, sensitive and reliable quality control method for the raw material has become a necessity. Ultra-high performance liquid chromatography coupled to mass spectrometry (UHPLC–MS) was investigated as an ultrafast, accurate, and sensitive method in the quantification of chromones (aloeresin A, aloesin) and anthrones (aloin A and B) in 101 *A. ferox* exudates. The method was validated for accuracy, precision and limits of detection and quantification. The calibration curves showed good linearity ( $R^2 > 0.99$ ) within the concentration range and the recoveries of the four analytes ranged from 100% to 114% with the relative standard deviation lower than 2%. The limits of detection and quantification for all compounds were within a range of 0.2–1.1  $\mu\text{g/ml}$ . Quantitative determination of the four biomarkers showed high levels of aloeresin A (129.0–371.6  $\mu\text{g/mg}$ ) and aloesin (111.8–561.8  $\mu\text{g/mg}$ ) while aloin A (21.3–133.4  $\mu\text{g/mg}$ ) and aloin B (18.4–149.7  $\mu\text{g/mg}$ ) were present in lower amounts. Hierarchical cluster analysis revealed two major groups within the dataset. However geographical locality was not a major factor in the clustering observed. This validated, ultra-fast method for simultaneous quantification of chromones and anthrones present in *A. ferox* is recommended for routine quality control analysis.

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## 1. Introduction

*Aloe ferox* Mill. (Xanthorrhoeaceae) known as “bitter aloe” or “Cape aloe” is one of the most important *Aloe* species rivalled only by *Aloe vera* in terms of commercial and medicinal importance. This polymorphic species is indigenous to South Africa and occurs in the region of Swellendam in the Western Cape from where it extends along the coast to KwaZulu-Natal in the East (Fig. 1) (O’Brien et al., 2011). It is an arborescent perennial plant with a robust single stem (2–3 m in height) and is characterised by bright red, orange or rarely white or orange flowers, crowned by a large

rosette of numerous leaves which are glaucous and oval-lanceolate in a spiral arrangement (Van Wyk and Smith, 2014).

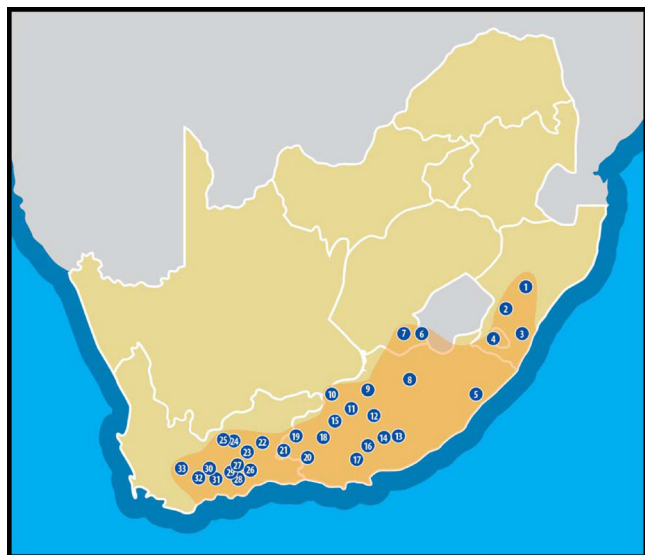
*Aloe ferox* is one of the most cited *Aloe* species and mainly wild-harvested in South Africa. Its laxative and cathartic effects led to its adoption by colonists in the Cape of Good Hope and exported it to Europe in the late eighteenth century (Grace et al., 2008). Due to its widespread healing properties, it has become a highly sought after medicinal plant both locally and internationally. The dried exudate, known commercially as Cape aloes, aloe bitters or aloe lump, is supplied largely in unprocessed form to international export markets in Europe and Asia (Cousins and Witkouski, 2012; Chen et al., 2012). The antimicrobial, antiviral, anti-fungal, antitumor and anti-inflammation activities of Cape aloes have been confirmed in several in vitro studies (Van Wyk and Wink, 2004; Kametani et al., 2007; Kambizi et al., 2007).

The phytochemistry of *A. ferox* is well studied with the anthrones (aloin A and B) and the chromones (aloesin and aloeresin A) (Fig. 2) being the most important constituents of the

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**Fig. 1.** Natural distribution of *Aloe ferox* in South Africa (orange). 1 = Otto's Bluff, 2 = Umzimkhulu, 3 = Oribi Flats, 4 = Mount Ayliff, 5 = Bashee River Valley, 6 = Lady Grey, 7 = Aliwal North, 8 = Queenstown, 9 = Cradock, 10 = Pearston, 11 = Cookhouse, 12 = Addo, 13 = Grahamstown, 14 = Alicedale, 15 = Graaff Reinett, 16 = Rocklands, 17 = Seweweekspoort, 18 = Jansenville, 19 = Steytlerville, 20 = Uniondale, 21 = Willowmore, 22 = Joubertina, 23 = Herbertsdale, 24 = Bokdrif, 25 = Stormsvlei, 26 = Albertinia district (Skadudal), 27 = Gouritz River Mouth, 28 = Vermaaklikheid, 29 = De Rust, 30 = Die Poort, 31 = Malgas, 32 = Hartenbosch, 33 = Heidelberg.

drug (Saccu et al., 2001). The anthrones are mainly responsible for the purgative properties of *A. ferox* (Coran et al., 2011), while aloesin and aloeresin A have been reported to exhibit skin lightening effects (Zahn et al., 2008). The aforementioned compounds, together with *p*-coumaric acid, are considered as biomarkers for *Aloe* species (Rizzo et al., 2011). However, geographical distribution and other environmental factors have been reported to influence the chemical profiles of *A. ferox*. Adams (2014) reported on variation in phytoconstituents of *A. ferox* collected from the Eastern Cape and the Western Cape Provinces of South Africa which affect the quality of the end product. According to the British Pharmacopoeia (1993), aloin content is considered an important quality indicator where a minimum of 18% is required to meet the standards for export. Aloin is widely used in small amounts as a bittering agent in alcoholic beverages. The level of aloin in aloe gel drinks and mixed fruit juices is generally limited to 10 parts per million or less. A sensitive and reliable method to detect and quantify low levels of the compound in complex matrices would therefore be useful. Recent advances in research

have encouraged the inclusion of multiple biomarkers (active or inactive) in the development of quality control protocols for herbal medicines. Thus, aloeresin A and aloesin, the two major constituents together with aloin A and aloin B can be used as biomarkers to develop a quality control protocols for *A. ferox*. HPLC methods have been previously described for the analysis of aloesin, aloeresin A, and barbaloin (aloins A and B) (Zonta et al., 1995; Park et al., 1998; Zahn et al., 2008). However, these methods were laborious and a shorter method may be desirable for routine quality control. In this study, we propose UHPLC–MS as a fast method for the simultaneous quantification of anthrones and chromones in *A. ferox* exudates.

## 2. Results and discussion

### 2.1. UHPLC–MS method validation

#### 2.1.1. Linearity

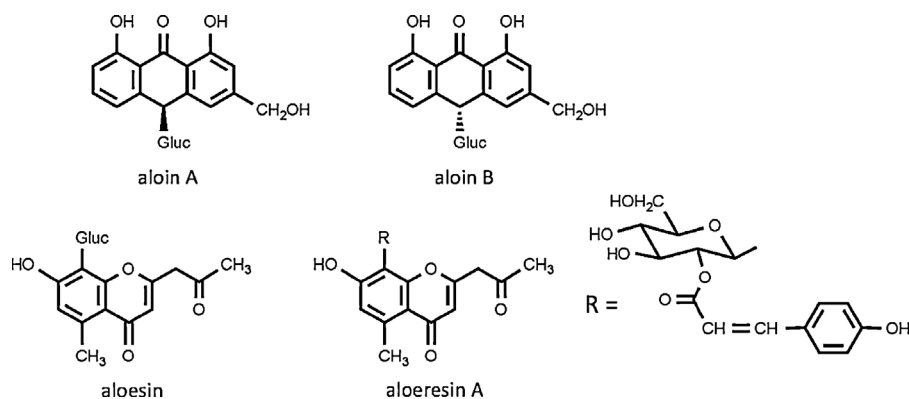
Calibration curves (based on the UV data) were developed to determine the coefficient of determination ( $R^2$ ) for each standard. The calibration curve was linear within the range of 0.5–100  $\mu\text{g}/\text{ml}$  for aloesin, aloeresin A, aloin A and B with  $R^2 > 0.99$  (Table 1).

#### 2.1.2. Precision, accuracy and LOD and LOQ

The LOD and LOQ values are shown in Table 1. The recoveries of the four standards ranged from 102% to 109% with the %RSD < 0.6%. Recoveries between 80% and 120% were reported to be acceptable for botanical compounds (Kamatou et al., 2012). The intra-day and inter-day precisions were found to be less than 0.7% and 1.2%, respectively which indicates the repeatability of the method (Table 2).

### 2.2. Quantitative analysis of *Aloe ferox*

The UHPLC–MS profiles of *A. ferox* exudate show minimal qualitative variation between samples obtained from different geographical localities. A UHPLC chromatogram of *A. ferox* is shown in Fig. 3, displaying the four biomarkers. The peak retention times and *m/z* values of the biomarkers are shown in Table 3. Quantitative variation of the samples was investigated by comparing the levels of the four biomarkers and the results are presented in Table 4. Aloin B content varied from 18.4 to 149.7  $\mu\text{g}/\text{mg}$ , while aloin A ranged from 21.3 to 133.4  $\mu\text{g}/\text{mg}$  of dried exudate. Based on the mean values of these two compounds, it can be concluded that the two anthrone glycosides are present in equal amounts as previously reported. Aloeresin A varied from 129.0 to 371.6  $\mu\text{g}/\text{mg}$  and aloesin from 111.8 to 561.8  $\mu\text{g}/\text{mg}$  which demonstrates that the two chromones occur in higher levels compared to aloin. Van Wyk et al. (1995) and Saccu et al. (2001)



**Fig. 2.** Chemical structures of the four main compounds of *Aloe ferox* leaf exudate.

**Table 1**Calibration equations, coefficient of determination ( $R^2$ ), limits of detection (LOD) and limits of quantification (LOQ) and recovery for the four standards.

| Compounds   | Calibration equation  | Coefficient of determination ( $R^2$ ) | LOD ( $\mu\text{g/ml}$ ) | LOQ ( $\mu\text{g/ml}$ ) | %Recovery | %RSD |
|-------------|-----------------------|--|--------------------------|--------------------------|-----------|------|
| Aloin A     | 15,663.3 * x + 726.8  | 0.9995                                 | 0.192                    | 0.640                    | 102.15    | 0.36 |
| Aloin B     | 10,753.2 * x + 1000.0 | 0.9983                                 | 0.321                    | 1.069                    | 108.76    | 0.35 |
| Aloeresin A | 18,728.8 * x + 171.3  | 0.9994                                 | 0.220                    | 0.627                    | 103.43    | 0.37 |
| Aloesin     | 9834.5 * x – 1022.2   | 0.9998                                 | 0.188                    | 0.734                    | 103.45    | 0.53 |

reported that *A. ferox* contains these four major compounds and the results are in agreement with those findings. Quantitative variation was noted not only for samples collected in different localities, but also for individual plants collected at the same location as demonstrated by the standard deviations (Table 4). The UHPLC–MS method was developed to simultaneously quantify the four biomarkers in all *A. ferox* samples within a very short analysis time (3 min).

### 2.3. Hierarchical clustering analysis

Hierarchical cluster analysis was performed with the objective of assessing dissimilarity patterns within the *A. ferox* data based on the levels of the four marker constituents. The results were visualised on a dendrogram, calculated using Ward's minimum variance method and sorted according to the index where clusters with higher observation indices are grouped on the far right (Fig. 4a). The dendrogram shows several branches formed as a function of the vertical coordinate, which represents an increase in variance. Two large clusters are however more pronounced of highest variance and these have been clearly indicated by the different colours (blue and green) (Fig. 4a). To investigate the observations associated with this clustering pattern, the corresponding scores plot of the observations was constructed (Fig. 4b). The scores plot of the first and second principal components (PC1 vs PC2) shows that the greatest variation (56%) is observed along PC1 which contributes to the separation of samples into two clusters corresponding to the two distinct branches in the dendrogram. The two clusters do not follow a specific geographical pattern as they both contain samples from all three Provinces namely; Eastern Cape (EC), Western Cape (WC) and KwaZulu-Natal (KZN). Separation along PC2 shows most observations within the blue cluster distributing along the positive and negative PC2 with some observations falling outside of the Hotellings'  $T^2$  ellipse. The 32% variation along PC2 can therefore be attributed mainly to sub-clustering within the blue cluster which is also evident as multiple sub-branching in the dendrogram (Fig. 4).

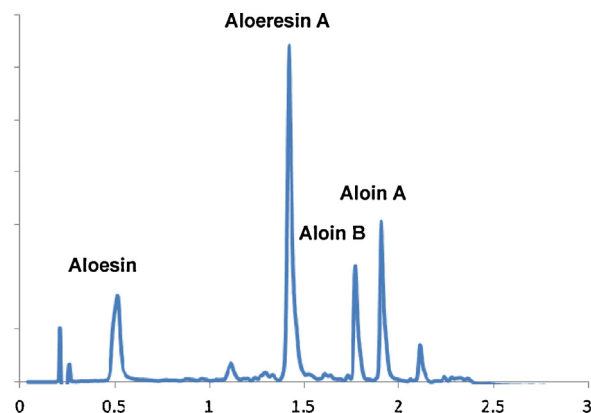
**Table 2**Precision of the developed method ( $n=3$ ).

| Standard    | Concentration ( $\mu\text{g/ml}$ ) | Intra-day RSD (%) | Inter-day precision RSD (%) |
|-------------|------------------------------------|-------------------|-----------------------------|
| Aloin A     | 10                                 | 0.00              | 0.72                        |
|             | 50                                 | 0.60              |                             |
|             | 100                                | 0.21              |                             |
| Aloin B     | 10                                 | 0.57              | 1.20                        |
|             | 50                                 | 0.25              |                             |
|             | 100                                | 0.59              |                             |
| Aloeresin A | 10                                 | 0.57              | 0.60                        |
|             | 50                                 | 0.29              |                             |
|             | 100                                | 0.00              |                             |
| Aloesin     | 10                                 | 0.55              | 0.27                        |
|             | 100                                | 0.25              |                             |

Following evidence of two existing clusters in the *A. ferox* data, it was necessary to investigate the discriminating variables. A loadings scatter plot (P1 vs P2) was constructed to show how the X-variables (marker compounds) correlate to each other as well as how they may influence the differences observed in the scores plot (Fig. 5). The loadings plot shows that aloeresin A and aloesin are positively correlated thus an increase in one compound will result in an increase in the other and vice versa. The same correlation pattern was observed for aloin A and B where these two compounds also show positive correlation due to their proximity in the plot. In contrast however; the compound pairs (aloin A and B, aloeresin A and aloesin) occur on opposite sides of the loadings plot, implying that the pairs are negatively correlated. The occurrence of one combination in high levels therefore influences the presence of the other combination in lower levels and vice versa.

The loadings plot was also used to explain the observed clusters in the scores plot. Aloeresin A and aloesin on the positive P1 loading are positively correlated to samples on the positive PC1 of the scores plot (green cluster). Conversely, aloin A and B on the negative P1 loading are positively correlated to samples on the negative PC1 in the score plot (blue cluster). To better explain the observed correlation, the group to average comparison for each cluster was performed and the corresponding contribution plots are displayed in Fig. 6. The vertical scale corresponds to the scaling of each variable and in units of standard deviations. The dominating blue bars observed (aloin A and B) indicate the greatest positive deviation of samples in the blue cluster from the model average with respect to the two marker molecules. An inspection of the aloin B variable plot showed that the majority of samples in the blue cluster contained higher than average (76.09  $\mu\text{g/mg}$ ) levels of aloin B and a similar pattern was observed for aloin A (Fig. 6a).

The contribution plot for the group to average comparison of the green cluster samples was also constructed (Fig. 6b). The plot shows positive deviation for aloeresin A and aloesin which is correlated to higher levels of the two compounds in the green

**Fig. 3.** Typical UHPLC chromatogram of *Aloe ferox* leaf exudate.

**Table 3**  
Mass and retention times of characterised chromones and anthrones.

| Peak number | Retention time (min) | Compound    | Molecular weight | Molecular ion           |
|-------------|----------------------|-------------|------------------|-------------------------|
| 1           | 0.53                 | Aloesin     | 394              | 395[M+H] <sup>+</sup>   |
| 2           | 1.44                 | Aloeresin A | 540              | 541[M+H] <sup>+</sup>   |
| 3           | 1.82                 | Aloin B     | 418              | 239[M–179] <sup>+</sup> |
| 4           | 1.93                 | Aloin A     | 418              | 239[M–179] <sup>+</sup> |

cluster compared to the model average (246 µg/mg and 296 µg/mg for aloeresin A and aloesin, respectively). The amount of aloesin in the green cluster is evidently more than aloeresin A and hence much of the observed difference is attributed to aloesin. The negative deviation of both aloin A and B from the average demonstrates that both quantities occur in lower levels compared to the model average.

### 3. General experimental procedures

#### 3.1. Sample collection and reagents

*Aloe ferox* plants in their natural habitat were collected from 34 localities in three Provinces (Eastern Cape, Western Cape and KwaZulu-Natal) of South Africa ( $n = 101$ ) (Fig. 1, Table 4). All solvents and chemicals were of analytical or HPLC grade (Merck Ltd, Germany). Water was purified by a Milli-Q-system (Millipore Corporation, USA). The standards: aloin A, aloin B and aloeresin A

were purchased from Sigma–Aldrich (Germany) and aloesin (purity  $\geq 99\%$ ) was isolated in our laboratory using high-performance counter current chromatography (HPCCC). The identity and purity of the compound was confirmed by NMR and UHPLC–MS.

#### 3.2. Sample preparation

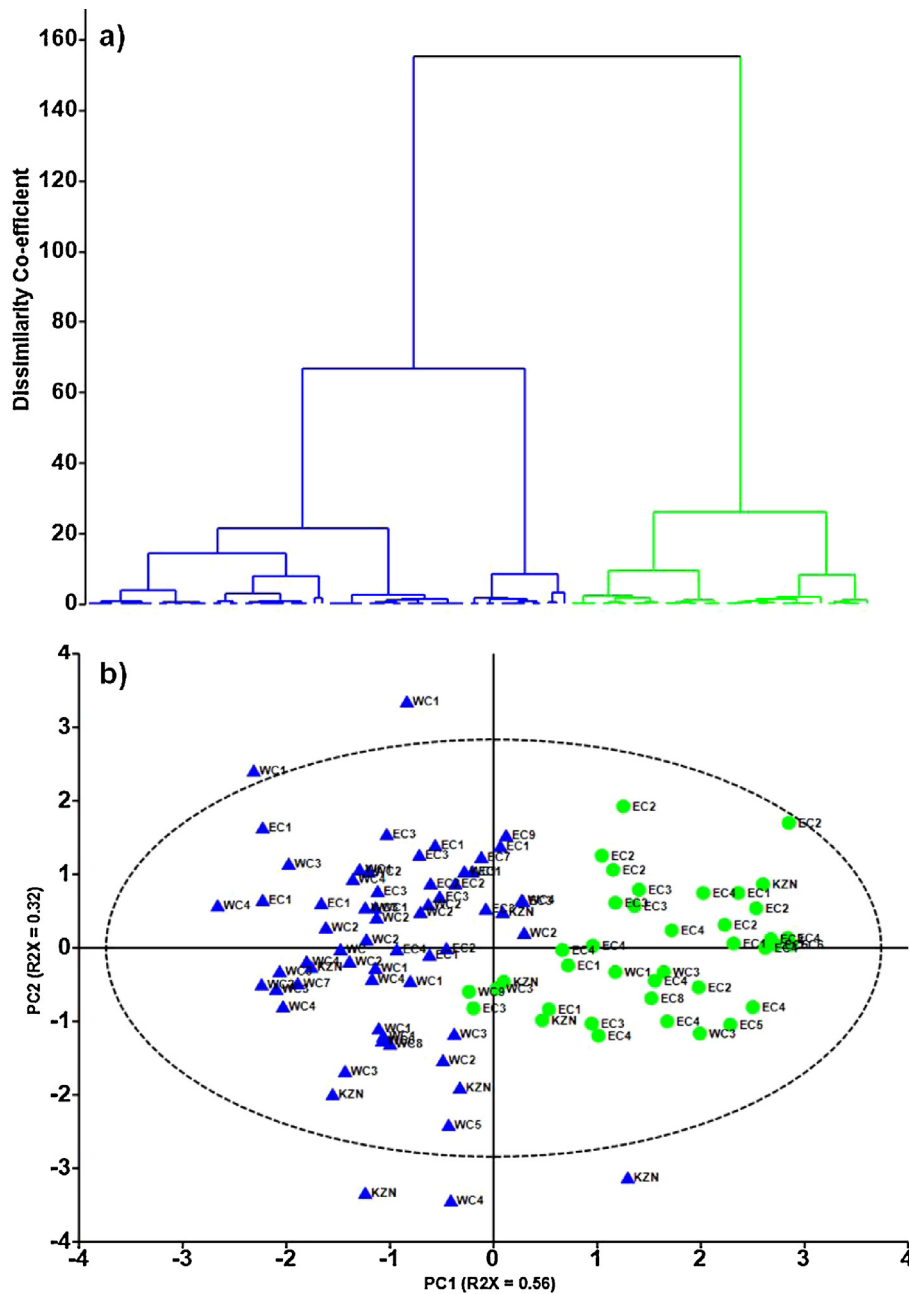
The tip of a mature leaf was excised and the exudates allowed to drip onto a piece of filter paper. The rapidly air-dried filter paper was transferred to a vial and covered with methanol to extract the exudate. The process was repeated three times and the methanol evaporated to concentrate the exudate. The dried exudates were diluted to a concentration of 1 mg/ml with methanol (HPLC grade), filtered and transferred to UHPLC–MS vials for analysis.

#### 3.3. UHPLC–MS analysis

The Waters Acquity Ultra High Performance Liquid Chromatographic system coupled with a PDA detector (Waters, USA) and XevoG<sub>2</sub>-Tof MS (Waters, USA) were used for this study. The separation was achieved on an Acquity UPLC BEH C<sub>18</sub> column (50 mm × 2.1 mm, i.d., 1.7 µm particle size, Waters) at a flow rate of 0.6 ml/min. The mobile phase consisted of 0.1% formic acid in water (A) and acetonitrile (B) with a linear gradient elution as follows: initial ratio 90% A: 10% B, changed to 68% A: 32% B in 2.5 min, and back to initial ratio in 0.5 min. The column temperature was set at 40 °C and the injected volume was 2 µl. ESI source was operating in positive mode and nitrogen was used as the desolvation gas. Data were acquired between 100 and 1000

**Table 4**  
Quantitative analysis of major constituents of *A. ferox* from different locations (several samples per location were used and results expressed as mean ± SD, µg/mg of dried exudate).

| Specimen number | Localities             | Aloesin       | Aloeresin A  | Aloin A      | Aloin B      |
|-----------------|------------------------|---------------|--------------|--------------|--------------|
| 1               | Otto's Bluff           | 167.7 ± 57.4  | 180.0 ± 21.2 | 77.4 ± 19.3  | 86.6 ± 25.4  |
| 2               | Umzimkhulu             | 228.1 ± 75.3  | 140.9 ± 75.8 | 49.9 ± 25.0  | 58.6 ± 28.8  |
| 3               | Oribi Flats            | 301.2 ± 36.4  | 289.6 ± 81.5 | 57.6 ± 18.6  | 56.5 ± 23.9  |
| 4               | Mount Ayliff           | 250.9 ± 16.4  | 249.5 ± 42.1 | 90.8 ± 27.3  | 98.0 ± 32.9  |
| 5               | Bashee River Valley    | 316.8 ± 66.2  | 241.1 ± 8.2  | 68.9 ± 19.1  | 66.8 ± 25.0  |
| 6               | Lady Grey              | 304.5 ± 21.0  | 239.0 ± 48.3 | 99.1 ± 17.6  | 108.9 ± 20.9 |
| 7               | Aliwal North           | 446.8 ± 105.9 | 233.8 ± 32.5 | 67.7 ± 20.7  | 69.1 ± 25.3  |
| 8               | Queenstown             | 345.7 ± 10.4  | 281.8 ± 45.1 | 73.9 ± 22.5  | 75.3 ± 30.0  |
| 9               | Cradock                | 297.5 ± 68.7  | 260.4 ± 22.4 | 97.3 ± 10.6  | 104.3 ± 16.8 |
| 10              | Pearston               | 366.9 ± 102.0 | 266.9 ± 12.8 | 97.7 ± 28.2  | 72.4 ± 32.7  |
| 11              | Cookhouse              | 446.3 ± 73.1  | 298.3 ± 29.8 | 65.4 ± 15.0  | 64.2 ± 20.8  |
| 12              | Addo                   | 425.9 ± 37.9  | 299.2 ± 75.9 | 45.6 ± 6.7   | 30.7 ± 8.3   |
| 13              | Grahamstown            | 338.3 ± 65.4  | 301.3 ± 65.1 | 48.9 ± 11.2  | 40.1 ± 11.8  |
| 14              | Alicedale              | 405.3 ± 35.6  | 299.6 ± 12.9 | 35.7 ± 3.1   | 20.8 ± 0.5   |
| 15              | Graaff-Reinett         | 330.9 ± 51.6  | 287.9 ± 13.9 | 85.8 ± 18.9  | 88.0 ± 31.0  |
| 16              | Rocklands              | 306.2 ± 47.8  | 248.6 ± 14.8 | 47.5 ± 16.3  | 39.6 ± 20.1  |
| 17              | Seweweekspoort         | 301.5 ± 119.8 | 228.3 ± 9.0  | 54.0 ± 17.6  | 42.6 ± 19.7  |
| 18              | Jansenville            | 316.8 ± 31.6  | 295.0 ± 3.2  | 80.4 ± 17.3  | 77.0 ± 22.0  |
| 19              | Steytlerville          | 395.0 ± 31.7  | 295.2 ± 5.5  | 48.0 ± 9.5   | 38.5 ± 11.9  |
| 20              | Uniondale              | 212.6 ± 91.2  | 209.9 ± 66.7 | 69.5 ± 13.2  | 74.0 ± 12.6  |
| 21              | Willowmore             | 265.5 ± 17.5  | 263.6 ± 32.9 | 75.3 ± 22.8  | 78.2 ± 29.5  |
| 22              | Joubertina             | 383.4 ± 13.9  | 246.0 ± 48.7 | 44.4 ± 14.3  | 41.0 ± 18.3  |
| 23              | Herbertsdale           | 189.9 ± 34.0  | 245.9 ± 47.8 | 87.5 ± 22.3  | 88.4 ± 18.2  |
| 24              | Bokdrif                | 182.1 ± 36.8  | 255.1 ± 49.2 | 95.9 ± 8.5   | 100.5 ± 3.7  |
| 25              | Stormsvlei             | 210.8 ± 59.8  | 225.8 ± 24.2 | 107.1 ± 5.7  | 95.6 ± 6.9   |
| 26a             | Albertinia (Skadudal)  | 292.4 ± 41.5  | 282.1 ± 47.8 | 106.7 ± 24.1 | 114.1 ± 32.5 |
| 26b             | Albertinia (Onverwagt) | 280.6 ± 136.4 | 253.5 ± 85.0 | 98.0 ± 23.8  | 101.3 ± 23.3 |
| 27              | Gouritz River Mouth    | 316.5 ± 25.7  | 231.5 ± 20.9 | 84.8 ± 12.8  | 96.1 ± 15.6  |
| 28              | Vermaaklikheid         | 172.3 ± 61.1  | 169.4 ± 54.0 | 88.9 ± 37.9  | 96.9 ± 45.0  |
| 29              | De Rust                | 266.1 ± 57.3  | 173.2 ± 22.3 | 86.0 ± 16.5  | 92.0 ± 18.8  |
| 30              | Die Poort              | 266.3 ± 22.0  | 239.9 ± 30.2 | 84.9 ± 16.8  | 94.9 ± 19.0  |
| 31              | Malgas                 | 248.4 ± 37.8  | 189.4 ± 35.1 | 97.6 ± 14.7  | 101.1 ± 12.1 |
| 32              | Hartenbosch            | 308.2 ± 54.3  | 233.9 ± 21.9 | 82.3 ± 27.2  | 99.1 ± 33.2  |
| 33              | Heidelberg             | 167.4 ± 52.2  | 223.2 ± 53.6 | 103.5 ± 14.4 | 99.1 ± 24.3  |
|                 | Average                | 296.0 ± 79.5  | 245.8 ± 41.7 | 75.8 ± 20.4  | 75.4 ± 24.9  |
|                 | Range ( $n = 101$ )    | 111.8–561.8   | 129.0–371.6  | 21.3–133.4   | 18.4–149.7   |



**Fig. 4.** (a) Dendrogram showing dissimilarity patterns of *Aloe ferox* exudates ( $n = 101$ ) based on aloresin A, aloresin, aloin A and aloin B composition. (b) The corresponding scores plot shows observations coloured according to the two major clusters; green (●) and blue clusters (▲). (For interpretation of reference to color in this figure legend, the reader is referred to the web version of this article.)

$m/z$ . The capillary voltage and the sampling cone voltages were 3.8 kV and 35 V, respectively. The extraction cone was 4 V and the source temperature was 100 °C. Other parameters include the desolvation temperature (450 °C); the cone gas flow rate of 10 l/h with a desolvation gas flow of 550 l/h. The chromatographic software MassLynx 4.1 (Waters, USA) was used for data acquisition and processing.

#### 3.4. UHPLC–MS method validation

##### 3.4.1. Calibration curves

UHPLC–MS calibration curves (linear regression) were developed for the four marker compounds. A concentration range of 0.5–100  $\mu\text{g/ml}$  was used and the calibration curves were

generated by plotting the peak area against the concentration for each analyte.

##### 3.4.2. Precision, accuracy, limits of detection and quantification

Intra-day and inter-day variations were determined to measure the precision of the method. For intra-day test, three different concentrations of standard solutions were analysed in triplicate within one day. For the inter-day test, the solutions were examined in triplicate for three consecutive days. Method accuracy was determined using standard spiking method where three concentrations (high, medium and low) within the calibration range were selected. A known amount of standard was added to the aloe extract samples. The samples were measured in triplicate and the recovery was calculated. The limits of detection (LOD) and



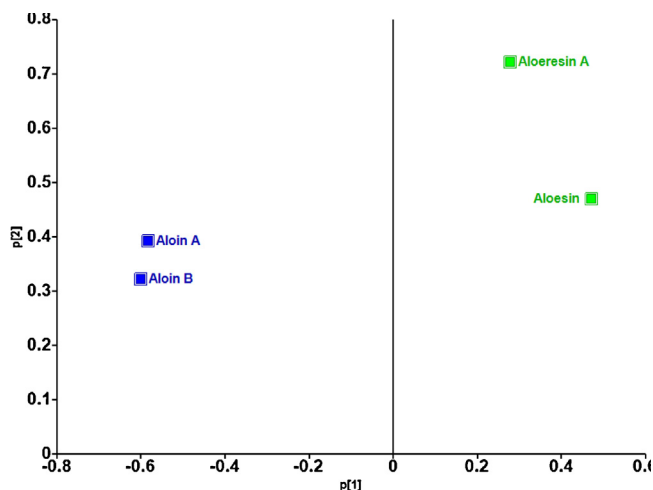


Fig. 5. A loading scatter plot demonstrating correlation between the marker compounds and their influence on the clustering pattern observed in the scores plot.

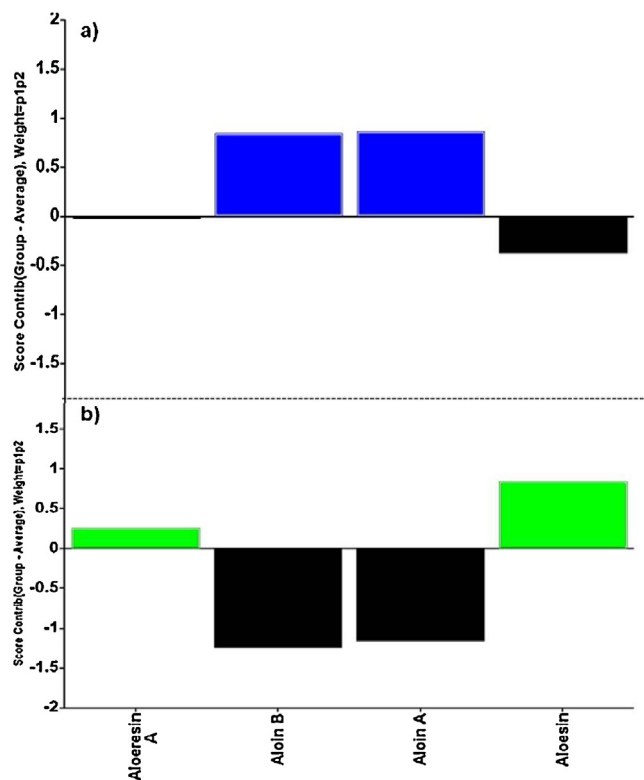


Fig. 6. (a) Contribution plots showing the group to average comparison of the blue cluster and (b) the green cluster.

quantification (LOQ) for the four standards were determined based on the standard deviation (SD) of the response and the slope, using  $LOD = 3.3(\sigma/S)$  and  $LOQ = 10(\sigma/S)$ ; where  $S$  is the slope of the calibration curve and  $\sigma$  is the SD of the response.

### 3.5. Data analysis

The amount of each analyte present in the samples was calculated from the calibration curve. Hierarchical clustering analysis (HCA) of the UHPLC data was also performed to evaluate the distribution of the four biomarkers in *A. ferox* exudates. The data were exported to SIMCA-P+ 13.0 software (Umetrics AB, Sweden) for analysis. A dendrogram and an interactive score scatter plot were constructed to show chemical relationships and geographical variations within the samples. The loadings and contribution plots were evaluated to identify variables that influence the clustering pattern observed in the dendrogram and the scores plot.

### Acknowledgment

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