Talanta 83 (2011) 1198–1208

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Dissimilar chromatographic systems to indicate and identify antioxidants from *Mallotus* species

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ARTICLE INFO

Article history:
Available online 19 November 2010

Keywords:
Fingerprints
Antioxidant activity
Dissimilarity
Multivariate calibration

ABSTRACT

The genus of *Mallotus* contains several species commonly used as traditional medicines in oriental countries. A data set containing 39 *Mallotus* samples, differing in species, cultivation conditions, harvest season and/or part of the plant was used to develop fingerprints on two dissimilar chromatographic systems. An exploratory analysis with principal component analysis (PCA) was performed on both data sets individually. The results were also combined to obtain additional information on the unknown samples included in the data set. Furthermore, the antioxidant activity of the samples was measured and modelled as a function of the fingerprints using the orthogonal projections to latent structures (O-PLS) technique. The regression coefficients of the models were studied to indicate the peaks potentially responsible for the antioxidant activity. The indicated peaks were analyzed and identified by HPLC coupled to mass spectrometry (HPLC–MS). Because of the complexity of biological samples, it was aspired to separate co-eluting components based on the significant difference in chromatographic selectivity on the dissimilar systems and consequently obtain additional, complementary information on the contribution of the individual components to the antioxidant activity. The results illustrate the potential use of dissimilar chromatographic systems. Several initially co-eluting compounds could be separated on the dissimilar system. The corresponding regression coefficients provided complementary information on the potential antioxidant activity of the separated compounds.

1. Introduction

Traditional medicines containing plant-, animal- and mineral-based products have an important role in the primary health care needs in many oriental countries. During the past decade, traditional medicines became more and more important in western countries for preventive and therapeutic purposes. As the use of traditional medicines becomes an increasingly important industry, their identity and quality becomes an important issue. To ensure the patient's safety it is extremely important to use traditional medicines which are identified and of which the quality is assessed [1,2].

In the conventional approach, just one or two marker constituents are considered to evaluate the quality of herbal medicines. Identification of just a few components hardly describes the complexity of herbal medicines, ignores synergistic interactions between the components and does not always allow assessing the total intrinsic quality of the herb. The concentrations of herbal components may vary significantly depending on, amongst others, the harvest season, the cultivation conditions and the drying processes [3]. Therefore, in general, quality control of herbal medicines by assaying just a few compounds is unreliable [4–8]. The World Health Organization (WHO) has introduced and accepted fingerprint analyses as a strategy for the assessment of herbal medicines [9]. A fingerprint obtained by, for instance, high-performance liquid chromatography (HPLC) characterizes the composition of the herbal medicine by means of a chromatogram representing all detectable chemical constituents, separated as much as possible. The obtained fingerprints can be used as a unique identification utility or to evaluate the authenticity of the herbal samples. Furthermore, chemometric treatment of the fingerprints allows modelling and predicting pharmacological activities (e.g. antioxidant and cytotoxic activities) and indicating the peaks potentially responsible for modelled activities [10–14].

One of the intensively studied herbal genera is the *Mallotus* genus (family *Euphorbiaceae*). Spread throughout South-East and North Asia, the genus comprises over 140 species of which many are used as traditional medicines [15]. The roots, stem barks, leaves

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doi:10.1016/j.talanta.2010.11.027

and fruits provide researchers with a broad basis in their search for new pharmaceutical active components [16]. Over the years, many studies on *Mallotus* species have been published and several pharmacologically active components were determined and isolated. The reported activities include anti-inflammatory, antioxidant, hepato-protective, cytotoxic, antimicrobial and retroviral ones [17–28]. Most of these studies focus on a very limited number of components analyzed in one particular species and hereby ignore the complex composition of the herbal samples, fail to discriminate between related species or the part of the plant used.

In an earlier paper [13], a data set containing the HPLC fingerprints of 39 *Mallotus* samples, differing in species, cultivation conditions, harvest time and/or part of the plant used, was analyzed. For all samples, the antioxidant activity was determined with a 1,1-diphenyl-2-picrylhydrazil (DPPH) [29] radical scavenging assay. The results were modelled as a function of the fingerprints using the orthogonal projections to latent structures (O-PLS) technique [30]. The regression coefficients of O-PLS then allowed indicating the peaks potentially responsible for the antioxidant activity of the samples. LC–MS analyses of the indicated peaks revealed four known and sixteen unknown components underlying the indicated peaks [14]. However, several peaks contained co-eluting components. As an alternative strategy to determine which peak belongs to an antioxidant component and which peak does not, an online HPLC analysis with photometric detection (e.g. with DPPH) in the post-column mode can be suggested. However, post-column detection methods requiring online derivatization are challenging and time-consuming to optimize and validate [31,32]. Even so, the technique would also suffer from the co-eluting compounds.

During the actual study, fingerprints of the *Mallotus* data set were developed on an additional dissimilar chromatographic system. Dissimilar or orthogonal systems are defined as 'systems that differ significantly in chromatographic selectivity'. With the dissimilar chromatographic systems it was aspired to separate the co-eluting components [33,34]. The newly obtained data set was also modelled using O-PLS and the regression coefficients were again studied to indicate the peaks potentially responsible for the antioxidant activity. Afterwards, LC–MS analyses were performed on the antioxidant samples to analyze the indicated peaks and reveal the underlying components. The results for the dissimilar systems were then compared in order to obtain complementary, additional information on the contribution of the individual components to the antioxidant activity of the samples.

2. Theory

2.1. DPPH radical scavenging assay

The DPPH radical scavenging assay [29] measures the capacity of a compound or a sample to scavenge the DPPH radical, DPPH• (1,1-diphenyl-2-picrylhydrazil). DPPH• has an absorption band at 515 nm which disappears upon reduction. The remaining absorbance at 515 nm is then inversely correlated to the antiradical activity of the compound or sample. In this study, the DPPH radical scavenging assay was performed on the 39 *Mallotus* samples to measure their antioxidant activity.

2.2. Data preprocessing

Before applying chemometric techniques, the chromatographic data needs to be organized in an $n \times p$ data matrix $\mathbf{X}$, where the n objects (herbal samples) constitute the rows and the p variables (time points) the columns.

The results of a chemometric technique are influenced by the applied data preprocessing methods. Different preprocessing methods are applied and compared. As the useful information resides in the between-sample variation of the variables, column centering is a generally applied preprocessing method. By removing the column mean from each corresponding value, a centered variable has a mean of zero. Other applied preprocessing techniques include normalization of the data to unit length (length one) and standard normal variate (SNV) [35–38].

Alignment or warping of chromatographic data also has an important place in the preprocessing of fingerprints. Along the time axis, peak shifts may occur due to instrument instability, column aging and small variations in mobile phase composition. Many techniques have been developed and amongst them correlation optimized warping (COW) [39–41], dynamic time warping [39], parametric time warping [41] and fuzzy warping [42] are the most important.

2.3. Exploratory analysis: principal component analysis

Principal component analysis (PCA) [35,36,38] defines new latent variables of which the first few contain most of the information of the data. It is a variable reduction technique which allows visualising the information included in the matrix. The latent variables, or principal components (PCs), are linear combinations of the original variables describing the largest remaining variation in $\mathbf{X}$. The different PCs are orthogonal and can be defined until a maximal number of PCs equal to $n-1$ (with $n<p$) is reached. The projections of the n objects from the original data space on a PC are the scores on this PC, while the contribution of each original variable to the score is reflected by its loading. Both scores and loadings can be used for exploratory analysis of the original data: a score plot representing the scores on two PCs gives information regarding the (dis)similarity of the objects, while a loading plot provides information on the contribution of the original variables to the considered PCs.

2.4. Orthogonal projections to latent structures

Orthogonal projections to latent structures (O-PLS) [30,36] is a linear multivariate calibration technique. It studies the relationship between an $n \times p$ data matrix $\mathbf{X}$ and an $n \times 1$ response vector $\mathbf{y}$. In linear multivariate calibration [35,36,38], the relationship between $\mathbf{X}$ and $\mathbf{y}$ can be described as:

$$\mathbf{y} = \mathbf{Xb} + \mathbf{f} \tag{1}$$

where $\mathbf{b}$ represents a $p \times 1$ vector of regression coefficients that expresses the contribution of the variables to the final model, and $\mathbf{f}$ the $n \times 1$ residual vector containing information that is not explained by the regression coefficients.

O-PLS is a modification to partial least squares [35,36,38], a linear multivariate calibration technique expressing the relationship between $\mathbf{X}$ and $\mathbf{y}$ by linear combinations of the original variables, latent variables called PLS factors, which maximizes the covariance between $\mathbf{X}$ and $\mathbf{y}$. Once these latent variables are calculated, the optimal number of PLS factors in the PLS model can be determined by means of a cross-validation procedure (e.g. leave-one-out cross-validation) [35,36,38].

Contrary to PLS, O-PLS does not just maximize the covariance between $\mathbf{X}$ and $\mathbf{y}$, it also removes the data orthogonal to $\mathbf{y}$ from the original $\mathbf{X}$ data. Consequently, the original data is split into two data sets, one that contains the $\mathbf{y}$-relevant information and one with the orthogonal data.

The O-PLS model can be described as follows:

$$\mathbf{X} = \mathbf{TP}^{\mathrm{T}} + \mathbf{T}_{\mathrm{Yorth}}\mathbf{P}_{\mathrm{Yorth}}^{\mathrm{T}} + \mathbf{E} \tag{2}$$

$$\mathbf{y} = \mathbf{TP}^{\mathrm{T}}\mathbf{b} + \mathbf{f} = \mathbf{Tq} + \mathbf{f} \quad (3)$$

$$\mathbf{b} = \mathbf{Pq} \quad (4)$$

where **T** represents the orthonormal $n \times n$ score matrix for **X** and **y**, **P** the orthonormal $p \times n$ loading matrix representing the regression coefficients of **X** on **T**, $\mathbf{T_{Yorth}}$ the orthogonal $n \times n$ score matrix for **X** and **y**, $\mathbf{P_{Yorth}}$ its corresponding orthogonal $p \times n$ loading matrix, **E** the $n \times p$ residual matrix of **X**, **b** the $p \times 1$ vector of regression coefficient calculated, **q** the $n \times 1$ loading vector representing the regression coefficients of **y** on **T** and **f** the $n \times 1$ residual vector of **y**.

Removing the orthogonal information of the original data set reduces the number of latent variables in the O-PLS model to a single one, allowing an improved interpretability of the regression coefficients [30].

3. Experimental

3.1. Preparation of the herbal extracts

39 *Mallotus* samples, belonging to at least 17 different species, were collected in different Vietnamese regions (Table 1). Five samples were unidentified. Depending on the species and the applicable nature preservation laws to protect the forests, the leaves, roots and/or bark were used. For some species, samples were collected in different Vietnamese provinces or at different times. All samples were authenticated by Professor Nguyen Nghia Thin (Hanoi National University, Vietnam).

For the extracts, 2.5 g crude plant material was weighed and extracted three times with 25 mL methanol in an ultrasonic bath (Branson Ultrasonic Corporation, Connecticut, USA) at a temperature between 40° and 50° C for 1 h. The extract was filtered through a 240 nm pore size filter paper (Whatman, Hanoi, Vietnam) and evaporated at reduced pressure (60 Pa) and elevated temperature (50 °C). The obtained crude extract was divided over three sample tubes, i.e. one for the DPPH radical scavenging assay, one for HPLC and MS analysis, and one as a voucher specimen. The voucher specimens were deposited at the Institute of Natural Products Chemistry, Hanoi, Vietnam.

3.2. Reference compounds

Myricetin and kaempferol-3-O-L-rhamnosyl were provided by the Drugs Analysis and Pharmacognosy Unite (Université Catholique de Louvain, Brussels, Belgium); quercitrin was obtained from Sigma–Aldrich (St. Louis, MO); mallonanoside A and mallonanoside B were provided by the Institute of Natural Products Chemistry (Vietnamese Academy of Science and Technology, Hanoi, Vietnam).

3.3. Sample preparation

Samples for HPLC and MS analysis were prepared diluting 50.0 mg crude extract in 2.0 mL methanol. The mixture was shaken during 15 min at 250 rpm on a shaking bath (Edmund Bühler, Hechingen, Germany) and afterwards filtered through a 2 μm pore size filter (Schleicher & Schuell, Dassel, Germany) followed by filtration trough a 25 mm syringe polypropylene membrane with 0.2 μm pore size (VWR International, Leuven, Belgium).

For the standards mallonanoside A, mallonanoside B, quercitrin, myricetin and kaempferol-3-O-L-rhamnosyl, 1.0 mg was weighed and dissolved in 10.0 mL methanol. Then, the same procedure was followed as for the crude extracts.

3.4. DPPH radical scavenging assay

The antioxidant activity of the *Mallotus* samples was determined using the DPPH radical scavenging assay. In its radical form, the 1,1-diphenyl-2-picrylhydrazil radical (DPPH°) absorbs at 515 nm. The absorbance disappears upon reduction. The remaining DPPH° concentration in the reaction medium is then estimated from a calibration curve and is inversely correlated to the antioxidant activity of the samples. The percentage of remaining DPPH° (%[$DPPH^{\circ}_{rem}$]) is expressed as follows:

$$\%[DPPH^{\circ}_{rem}] = \frac{[DPPH^{\circ}_{20\ min}]}{[DPPH^{\circ}_{0\ min}]} \times 100$$

where [$DPPH^{\circ}_{0\ min}$] is the starting concentration of DPPH radicals, and [$DPPH^{\circ}_{20\ min}$] the remaining concentration after 20 min of incubation with the sample.

An aliquot (50 μL at a concentration of 20 μg/mL) of a MeOH solution containing sample or a positive control (tocopherol) was added to 2.5 mL of daily prepared DPPH° solution (25 μg/mL in methanol). Control tubes ([$DPPH^{\circ}_{0\ min}$]) were prepared by adding an equal volume (50 μL) of the solvent (methanol). The DPPH° concentration in the reaction medium was calculated from a linear calibration curve at concentrations ranging from 1 to 50 μg/mL. Twenty minutes after starting the reaction, the absorbance at 515 nm was measured on an Uvikon 933 spectrophotometer (Kontron, Chichester, UK). All experiments were performed in triplicate and the reported results are the averages and standard deviations of three independent measurements. The average standard deviation for the 39 *Mallotus* samples was found to be 6.7. The samples were considered to be highly antioxidant when the $\%DPPH_{rem}$ was below 30, intermediately when the $\%DPPH_{rem}$ was between 30 and 50, and inactive when the $\%DPPH_{rem}$ was higher than 50. These limits were defined arbitrarily for this specific data set.

3.5. HPLC

3.5.1. Equipment, chemicals and reagents

The experiments were performed on a Shimadzu Prominence HPLC system (Shimadzu, Tokyo, Japan) equipped with an auto sampler, vacuum degasser, quaternary pump, column oven and a photodiode array detector. All data was acquired and processed using LC solution (Shimadzu, Tokyo, Japan).

For the chromatographic analyses, two chromatographic systems were selected out of 46 tested, using the weighted pair group method using arithmetic averages (WPGMA) dendrogram described in Dumarey et al. [43]: (a) two coupled Chromolith™ Performance RP-18e (100 mm × 4.6 mm I.D.) with a Chromolith guard column RP-18e (5 mm × 4.6 mm I.D.) purchased from Merck (Darmstadt, Germany), and (b) a Zorbax Eclipse XDB-C8 (150 mm × 4.6 mm I.D., 5 μm) from Agilent Technologies (Waldbronn, Germany).

The mobile phases were prepared using HPLC grade acetonitrile (Fisher Scientific, Leicestershire, UK), trifluoroacetic acid (TFA) (Sigma–Aldrich, Steinheim, Germany), and MilliQ water obtained from a MilliQ water purification system (Millipore, Bedford, MA).

3.5.2. Chromatographic conditions

The mobile phase for both dissimilar systems consisted of (A) 0.05% TFA in MilliQ water, and (B) 0.05% TFA in ACN. Gradient elution was applied. The gradient program for the two coupled Chromolith™ Performance RP-18e with guard column was 5–20% B in 0–25 min, 20–95% B in 25–50 min and 95% B in the 50–60 min interval, and for the Zorbax Eclipse XDB-C8 5–30% B in 0–20 min, 30–95% B in 20–50 min and 95% B in the 50–60 min interval.

Table 1
The Mallotus samples with their voucher number, species, origin, collection time, used part of the plant and the DPPH scavenging activity results indicated. The highly antioxidant samples are marked in bold.

Sample	Voucher number	Species	Origin	Collection time	Part of plant	%DPPH$_{rem}$ (n = 3)	s
1	01	*Mallotus luchenensis*	Son La	July 2006	Leaves	82.0	12.1
2	02	*Mallotus microcarpus*	Son La	July 2006	Leaves	63.6	13.0
3	03	*Mallotus barbatus*	Son La	July 2006	Leaves	79.4	9.7
4	MA07	*Mallotus sp1*	Van Hoa	April 2006	Leaves	113.0	20.5
5	NT01	*Mallotus barbatus*	Hagiang	November 2006	Leaves	77.2	10.4
6	NT02	*Mallotus paniculatus*	Hagiang	November 2006	Leaves	82.2	5.5
7	NT03	*Mallotus metcalfianus*	Hagiang	November 2006	Leaves	51.1	14.6
8	MA01	*Mallotus apelta (Ma1)*	Tam Dao	July 2006	Leaves	94.5	0.4
9	MA02	*Mallotus apelta (Ma2)*	Tam Dao	December 2006	Leaves	92.5	3.3
10	MA03	*Mallotus paniculatus*	Tam Dao	April 2006	Leaves	58.4	5.4
11	SP4	*Mallotus sp2*	Langson	March 2006	Leaves	56.8	3.9
12	SP5	*Mallotus philippinensis*	Langson	March 2006	Leaves	98.9	12.7
13	MA11	*Mallotus macrostachyus*	Langson	March 2006	Leaves	75.7	2.2
14	MA12	*Mallotus microcarpus*	Quangbinh	March 2006	Leaves	83.1	2.0
15	MA13	*Mallotus pallidus*	Quangbinh	March 2006	Leaves	65.3	1.9
16	**MA14**	***Mallotus oblongifolius***	**Quangtri**	**March 2006**	**Leaves**	**6.7**	**0.3**
17	**MA15**	***Mallotus floribundus***	**Langson**	**November 2006**	**Leaves**	**6.4**	**0.2**
18	MA16	*Mallotus cuneatus*	Langson	November 2006	Leaves	86.9	3.2
19	**MA17**	***Mallotus cuneatus***	**Quangbinh**	**December 2006**	**Leaves**	**10.3**	**4.1**
20	MA18	*Mallotus sp3*	Quang tri	December 2006	Leaves	91.6	4.1
21	MA19	*Mallotus yunnanensis*	Lang Son	November 2006	Leaves	91.6	6.6
22	MA20	*Mallotus poilanei*	Ke Bang	March 2006	Leaves	90.5	7.0
23	MA22	*Mallotus hookerianus*	Dakrong	March 2006	Leaves	50.0	4.6
24	MA23	*Mallotus nanus*	Daclak	March 2006	Leaves	78.4	9.5
25	MA24	*Mallotus sp4*	Daclak	March 2006	Leaves	56.9	11.7
26	M25	*Mallotus oreophilus*	LaoCai	June 2006	Leaves	88.8	10.5
27	**MA28**	***Mallotus philippinensis***	**Cucphuong**	**December 2006**	**Leaves**	**22.3**	**10.0**
28	**MA29**	***Mallotus barbatus***	**Cucphuong**	**December 2006**	**Leaves**	**11.3**	**4.8**
29	MP31L	*Mallotus paniculatus*	VQG Pumat	September 2006	Leaves	73.5	8.5
30	MP3R	*Mallotus paniculatus*	VQG Pumat	September 2006	Roots	91.5	5.7
31	MP33L	*Mallotus paniculatus*	Bach Ma-TTH	October 2006	Leaves	81.5	3.8
32	MP34R	*Mallotus paniculatus*	Bach Ma-TTH	October 2006	Roots	83.5	6.6
33	MP35R	*Mallotus paniculatus*	Cucphuong	December 2006	Roots	79.8	2.9
34	MP36L	*Mallotus paniculatus*	Cucphuong	December 2006	Leaves	75.3	8.8
35	**MN37R**	***Mallotus nanus***	**VQG-Bachma**	**May 2006**	**Roots**	**12.2**	**1.7**
36	**MN37L**	***Mallotus nanus***	**VQG-Bachma**	**May 2006**	**Leaves**	**4.5**	**1.0**
37	**MN39C**	***Mallotus nanus***	**VQG-Bachma**	**May 2006**	**Bark**	**27.1**	**4.7**
38	M40L	*Mallotus sp5*	VQG Bavi	August 2006	Leaves	73.7	8.7
39	M41C	*Mallotus sp6*	VQG Bavi	August 2006	Bark	65.6	8.0

Furthermore, for both systems, the column temperature was 25 °C, the flow rate 1.0 mL/min, the injection volume 10 μl, and the detection wavelength 254 nm.

3.6. LC–MS

All experiments were executed on an Alliance HPLC (Waters, Milford, Massachusetts, US) equipped with an auto sampler and column oven. MS-detection was conducted using an ion trap LCQ-advantage system (Thermo Fisher Scientific, Waltham, Massachusetts, US) equipped with an APCI interface. All MS analyses were performed with a mass precision of 0.5 atomic mass units (amu). The MS acquisitions were performed in both positive and negative atmospheric pressure ionisation modes.

The following APCI inlet conditions were used. Nitrogen was used both as a nebulising gas at 450 °C and an arbitrary flow of 70, and as a drying gas at 450 °C and an arbitrary flow of 30. The capillary temperature was set at 200 °C. In the positive mode, the capillary voltage was set to 26 V, the source voltage to 6 kV and the source current to 5 μA. In the negative mode, the capillary voltage was set to −4 V, the source voltage to 4.5 kV and the source current to 80 μA. In both modes 25 V of collision energy was applied.

3.7. Data analysis

Computations were performed on a PC with an Intel Core 2 Duo E6750 processor containing 2 gigabyte RAM and running Microsoft Windows XP Pro and Matlab™ 7.1 (The Mathworks, Natick, MA). All data (pre)processing were performed using m-files written for Matlab 7.1.

4. Results and discussion

4.1. DPPH radical scavenging test

The results (Table 1) show that only 8 of the samples are considered to have a high antioxidant activity (%DPPH$_{rem}$ < 30), i.e. *Mallotus oblongifolius* (16), *Mallotus floribundus* (17), *Mallotus cuneatus* (19), *Mallotus philippinensis* (27), *Mallotus barbatus* (28), and three *Mallotus nanus* samples (35–36–37).

Mallotus philippinensis and *Mallotus barbatus* both have antioxidant and non-antioxidant samples, with the antioxidant samples having their origin in Cucphuong. For *Mallotus nanus*, only the three samples (roots, leaves and bark) with origin in Bachma posses a high antioxidant activity, while those from Daclak did not. Furthermore, the data set contains six unknown samples (4–11–20–25–38–39), which did not show a high antioxidant activity.

4.2. Selection of dissimilar chromatographic systems

Two dissimilar systems were selected from [43] and used to develop the HPLC fingerprints of the *Mallotus* samples. The first system consisted of two coupled Chromolith™ Performance RP-18e columns and a Chromolith guard column RP-18e with water/acetonitrile at low pH as mobile phase and was selected based on previous fingerprint optimization [13]. The second sys-

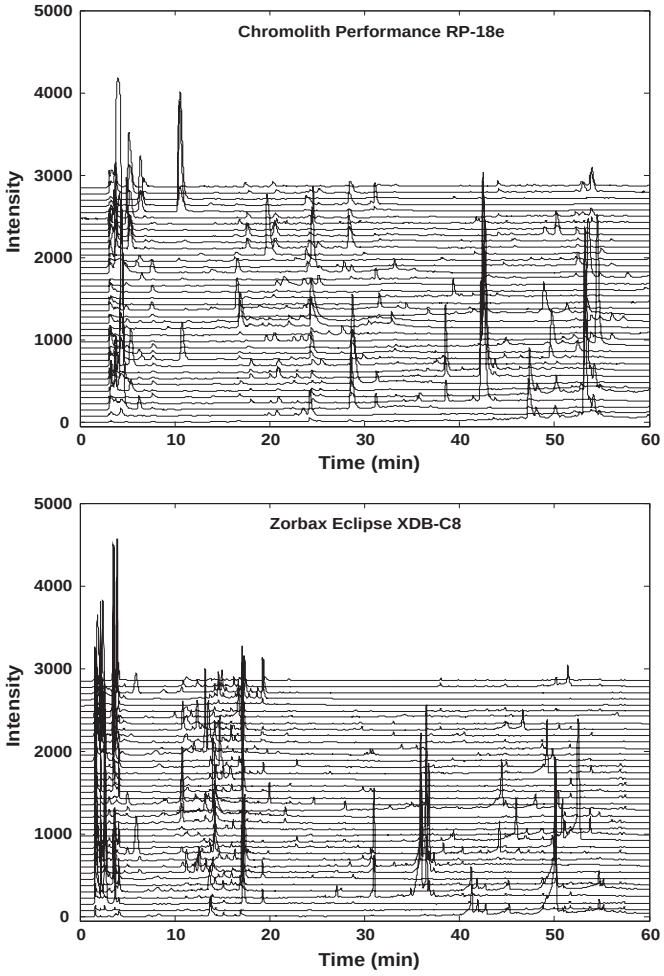


Fig. 1. 60 min fingerprints of the *Mallotus* extracts on the Chromolith Performance RP-18e columns (top) and the Zorbax Eclipse XDB-C8 column (bottom).

tem was selected out of 46 available at the laboratory of which their dissimilarity with the first system was visualized earlier using the weighted pair group method using arithmetic averages (WPGMA) dendrogram [43].

Initially several chromatographic systems were selected. All systems combined a mobile phase consisting of water and an organic modifier at low pH with the following columns: Zirchrom PS, Zirchrom PBD, Betasil Phenyl Hexyl and Zorbax Eclipse XDB-C8. Their separation ability for the *Mallotus* species was experimentally tested. Each column was injected with 10 μl of sample prepared as described in Section 3.3. Based on the highest number of peaks observed, the Zorbax Eclipse XDB-C8 column with water/acetonitrile at low pH as mobile phase was selected as second system.

4.3. HPLC fingerprints

The optimized experimental conditions for both chromatographic systems are described in Section 3.5.2. At least 17 different *Mallotus* species, of which the fingerprints are very different (Fig. 1), are present (Table 1). Therefore, it is most likely that different pharmaceutical activities can be attributed to different species. Due to the dissimilarity of both systems, the fingerprints recorded for the same sample also are very different. Prior to data treatment, baseline correction was performed on the individual chromatographic profiles by subtracting a blank.

4.4. Data treatment

4.4.1. Data preprocessing

Alignment or warping of chromatograms has an important place in the preprocessing of fingerprints to correct for the shifts in retention times that are observed between the chromatograms on a given system. However, proper alignment of the actual very diverse fingerprints could not be achieved. Seemingly corresponding peaks did not always contain identical compounds. Aligning data sets consisting of fingerprints of great diversity from complex biological samples requires additional information such as from a Diode Array detector or LC–MS data. As the LC–MS data of the antioxidant samples partially was unavailable, it was chosen to work with the unaligned data (as in [14] already was done).

Both data sets were preprocessed by column centering, normalization followed by column centering and standard normal variate followed by column centering prior to building the O-PLS model. Normalization followed by column centering gave the best results for these specific data sets. All data further discussed is preprocessed accordingly.

4.4.2. Exploratory analysis: principal component analysis

PCA has been applied on the individual data sets to verify whether groups of samples could be distinguished according to, for instance, the antioxidant activity, fingerprint profiles or species. Furthermore, it was evaluated if additional, complementary information could be extracted when the results of the PCA analysis on both data sets were compared.

For the monolithic columns, the PC1–PC2 score plot (Fig. 2a) reveals the samples with antioxidant activity (marked in bold) centrally situated. However, no clearly separated group of antioxidant samples could be distinguished. Combining the proximity of samples on the score plots and the a priori knowledge regarding both fingerprint profiles and species results in the distinction of three groups, i.e. (a) samples 4–8–9 (●), (b) samples 6–10–29–30–31–32–33–34–38–39 (■), and (c) samples 35–36–37 (▲). The remaining samples were labelled with **x** and cannot be further split into subgroups based on species or fingerprints. These groups allowed extracting information regarding three unidentified samples. Group (a) consists of two *Mallotus apelta* samples (8–9) and one unidentified sample (4). Because of the proximity on the score plot and the similar fingerprints profiles, it can be assumed that sample 4 belongs to the same species as samples 8 and 9. Similarly, group (b) consists of eight *Mallotus paniculatus* samples (6–10–29–30–31–32–33–34) and two unidentified samples (38–39). Also they most probably belong to the *Mallotus paniculatus* species. In addition, group (c) contains the three samples (35–36–37) belonging to the *Mallotus nanus* species.

The PC1–PC2 score plot from the second chromatographic system (Fig. 2b) reveals three samples with antioxidant activity clustered in group (d), i.e. samples 16–17–19 (⋆). The other active samples (marked in bold) are spread over the score plot and show no clustering tendency. Based on the knowledge of fingerprint profiles and species, group (a) containing samples 4–8–9 (●) again could be distinguished.

When evaluating the information from both chromatographic systems, the PC1–PC2 score plot of the monolithic column provided information on the unidentified samples 4, 38 and 39, while the Zorbax column also provided information on the unidentified sample 4. Furthermore, the plot from the monolithic column clustered the antioxidant *Mallotus nanus* samples and all *Mallotus paniculatus* samples, while the plot from the Zorbax column did not. The three samples of group (d) which are in each other's vicinity in Fig. 2a were more clearly isolated in Fig. 2b.

However, the PC plots do not show significantly diverging clustering tendencies between both systems. This could be expected,

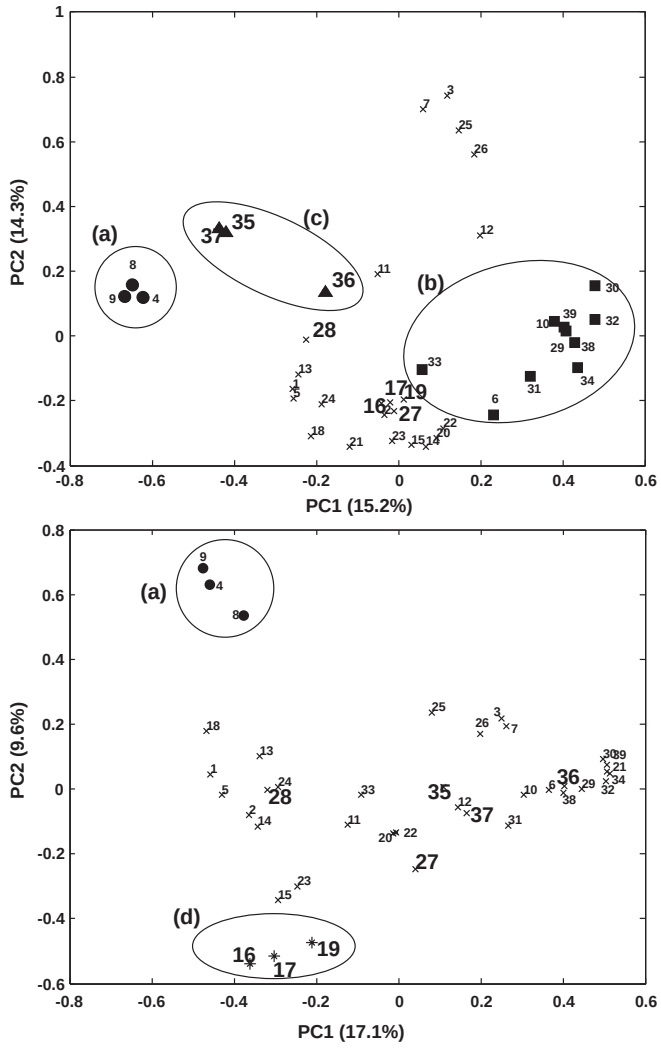


Fig. 2. PC1–PC2 score plot after normalization and column centering for the fingerprints of the 39 *Mallotus* samples on the monolithic system (top) and the Zorbax system (bottom). The highly active antioxidant samples are marked in bold. Groups (a), (b) and (c) are based on the proximity of samples on the score plots and the a priori knowledge regarding both fingerprint profiles, group (d) is a cluster of samples with high antioxidant activity separated on the second score plot.

as the clustering tendency on the score plots is based on the similarity of the fingerprint profiles and, even though measured on dissimilar systems, similar samples result in similar fingerprints within one chromatographic system. Nevertheless, the obtained results indicated that both chromatographic systems did provide some complementary information.

4.4.3. Orthogonal projections to latent structures

The main focus of this study is to indicate the peaks potentially responsible for the antioxidant activity of the *Mallotus* samples and to evaluate if dissimilar chromatographic systems lead to complementary information such as the separation of compounds co-eluting on one system and re-evaluate when an antioxidant compound was concerned. To indicate the potentially antioxidant peaks, a multivariate calibration model was constructed by applying the orthogonal projections to latent structures (O-PLS) technique to both data sets individually. The data matrix **X** consisted of the 39 fingerprints and the response vector **y** represented the results of the DPPH radical scavenging assay. The data was not split into a calibration and a test set because it was not large enough and prediction of the antioxidant activity of new samples is not our primary concern. The models were validated using leave-one-out cross validation and the model complexity was determined as a single component PLS model after removal of one orthogonal projection.

Table 2
Results from the DPPH radical scavenging assay and predictions from the models built with the 60 min fingerprints. Preprocessing: normalization and column centering.

Sample no.	%DPPH$_{rem}$	O-PLS (monolith)	O-PLS (Zorbax)
16	6.7	4.9	6.2
17	6.4	17.2	5.9
19	10.3	34.6	25.4
27	22.3	45.2	34.0
28	11.3	52.6	47.5
33	12.2	10.0	22.1
36	4.5	24.1	34.4
37	27.1	4.4	46.7

To evaluate the model's ability to predict the antioxidant activity of the species, and thus to indicate the peaks responsible for the activity, the prediction of the antioxidant activity for the samples was taken into account. For the monolithic column model the RMSECV was 14.1 and for the antioxidant samples, the model predicted samples 16, 17, 35, 36 and 37 as being highly antioxidant, samples 19 and 27 as intermediately active and sample 28 as inactive (but borderline). For the Zorbax column model the RMSECV was 14.3 and predicted samples 16, 17, 19 and 35 as highly active, and samples 27, 28, 36 and 37 as intermediately active (Table 2). None of both models predicted inactive samples to have an intermediate or high antioxidant activity.

To indicate the potential antioxidant compounds, the regression coefficients of both models were examined and the indicated chromatographic peaks were analyzed by LC–MS. Chromatographic peaks of potentially antioxidant compounds correspond to negative regression coefficient peaks as the DPPH radical scavenging test result decreased with increasing activity. The fingerprints of the highly active antioxidant samples on both systems were compared with the obtained regression coefficients (Fig. 3) and the chromatographic peaks corresponding to negative regression coefficient peaks were analyzed by LC–MS.

All MS analyses were performed in both the positive and the negative modes. Because of the presence of TFA as additive, many of the analyzed compounds are bound to TFA in the MS spectra when analyzing in the negative mode, causing a difference of +113 amu. During analyses in the positive mode, this problem does not occur. In the negative mode, all reported values in this paper are corrected for the addition of TFA to avoid confusion.

5. Identified compounds

LC–MS analysis of the negative regression peaks from the monolithic column identified four known compounds, i.e. mallonanoside A, mallonanoside B, quercitrin and kaempferol-3-O-L-rhamnosyl (Fig. 4).

The three samples of *Mallotus nanus* and sample 27 presented the same peaks at 6.5 and 10.5 min corresponding to fragments of mallonanoside B and mallonanoside A, respectively (Fig. 5). Both compounds are C-glycosyl benzoic acid analogues that were recently identified [44]. Little is known about their activity, but several studies show structure-related analogues to posses antioxidant activity [45–47]. Furthermore, the flavonoids quercitrin (28.3 min) and kaempferol-3-O-L-rhamnosyl (31.1 min) were identified in the three *Mallotus nanus* samples. Similarly, the same compounds could be identified in the corresponding samples on the Zorbax column: mallonanoside B at 3.5 min, mallonanoside A at 3.8 min, quercitrin at 17.1 min and kaempferol-3-O-L-rhamnosyl at 19.2 min. Their

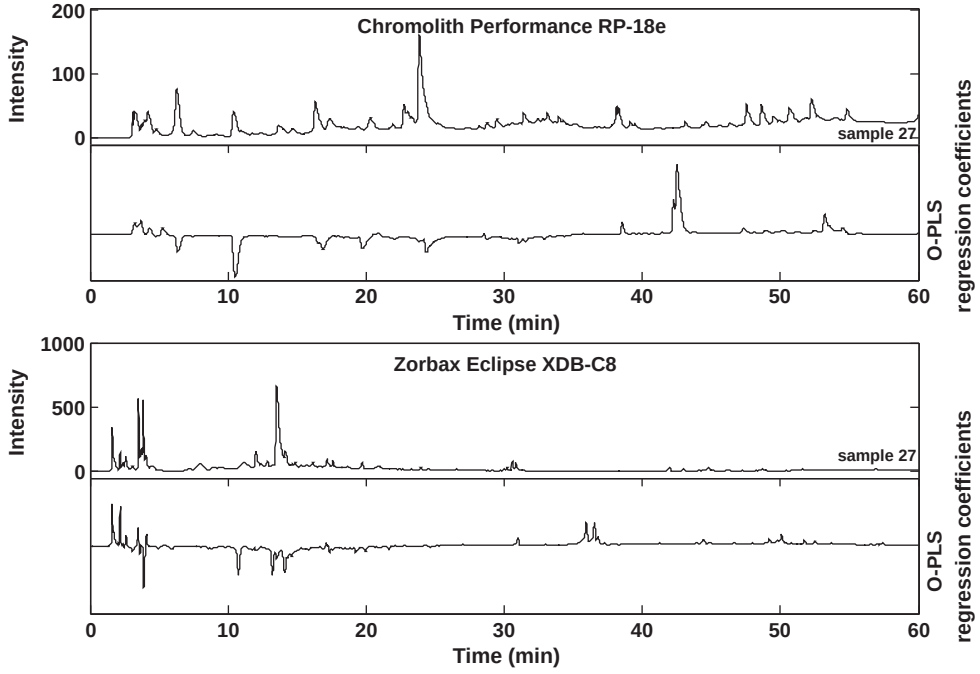
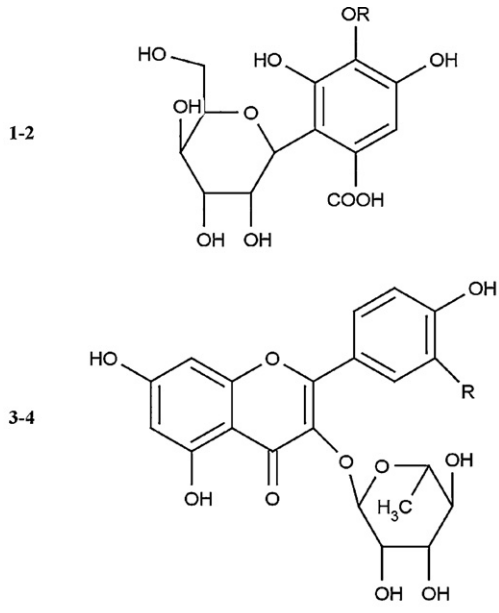


Fig. 3. Comparison of the fingerprints of the highly active antioxidant sample 27 and the obtained regression coefficients on both columns.



N°	Name	R	Molecular Weight (g/mol)
1	Mallonanoside A	CH3	346
2	Mallonanoside B	H	332
3	Quercitrin	OH	448
4	Kaempferol-3-O-L-rhamnosyl	H	432

Fig. 4. Structures of identified compounds.

chromatographic peaks on the fingerprints also corresponded to negative regression coefficients of the O-PLS model.

6. Unidentified compounds with important regression coefficients

Both systems show several peaks, corresponding to negative regression coefficients, which contain one or more unidentified compounds (Fig. 5). To simplify the discussion and to put the stress on the complementary information from both systems, only a brief description of the unidentified compounds is given. More information can be found in Table 3.

On the monolithic column in the negative mode, samples 16, 17, 19 and 27 have compounds A and B eluting at about 16.5 min, with compound B being a heteroside loosing 162 amu (hexose). Compound C can be found in the same samples eluting at about 23.5 min. It is most probably the aglycon of compound B, having 162 mass units less (corresponding to a hexose).

In the positive mode compounds E and F elute at 23.8 and 25.0 min, respectively, in sample, 37 and compound G at 25.0 min in samples 35 and 36. In both the positive and negative modes, compound H elutes at 24.5 min in samples 16, 17, 18 and 27, compound I at 19.7 min in sample 28 and compound J at 23.8 min in samples 35, 36 and 37.

For the Zorbax column, in the negative mode, samples 16, 17, 19 and 27 have compounds A and B eluting at about 10.7 min and compound C at about 13.1 min. Samples 16, 17 and 19 also have a compound K eluting at about 13.2 min, which is a splitted peak also containing compound C. In the positive mode compounds D and L elute at 14.3 min in samples 19 and 27, respectively, and compound E elutes at about 10.1 min in sample 37. In both the positive and negative modes, compound H elutes at about 14.1 min in samples 16, 17, 19 and 27, compound I elutes at 13.4 min in sample 28.

7. Extracting complementary information

The results of the LC–MS, discussed above, are compared. The main purpose was to verify whether the indicated compounds co-eluting on one system could be separated on the other. Furthermore, the regression peaks of the co-eluting compounds and of the corresponding separated compounds are discussed. A negative regression peak for co-eluting compounds provides no information on the contribution of the individual compounds to the antioxidant activity. Therefore, one must take into account the regression peaks of the separated compounds.

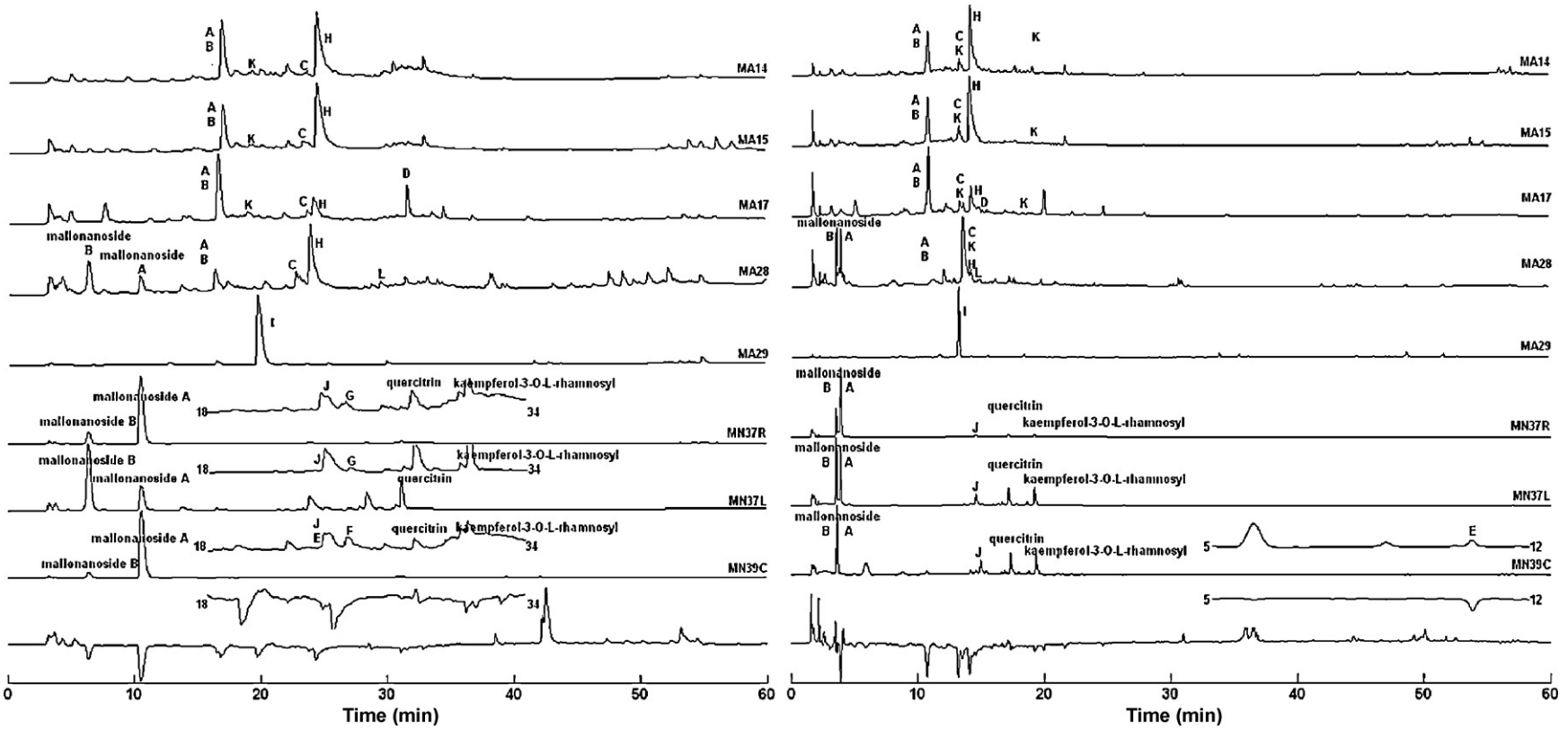


Fig. 5. Indication of the identified and unknown compounds. The fingerprints of the eight highly active antioxidant samples are plotted above the O-PLS regression coefficients of the models from the monolithic (left) and the Zorbax columns (right). The components are marked as described in the text.

Table 3
Results from the LC–MS analysis for the (A) monolithic (top table) and (B) Zorbax column. Compounds detected in the negative mode are marked with $^-$, compounds detected in the positive mode with $^+$. The corresponding mass values are put between brackets.

Retention time	Sample 16	Sample 17	Sample 19	Sample 27	Sample 28	Sample 35	Sample 36	Sample 37
(A) Monolithic column								
6.5 min				Mallonanoside B^{+-} ($333^+/331^-$)		Mallonanoside B^{+-} ($333^+/331^-$)	Mallonanoside B^{+-} ($333^+/331^-$)	Mallonanoside B^{+-} ($333^+/331^-$)
10.5 min				Mallonanoside A^{+-} ($347^+/345^-$)		Mallonanoside A^{+-} ($347^+/345^-$)	Mallonanoside A^{+-} ($347^+/345^-$)	Mallonanoside A^{+-} ($347^+/345^-$)
16.8 min	A^- (633^-)	A^- (633^-)	A^- (633^-)	A^- (633^-)				
	B^- (463^-)	B^- (463^-)	B^- (463^-)	B^- (463^-)				
19.5 min	K^- (305^-)	K^- (305^-)	K^- (305^-)					
19.8 min					I^{+-} ($360^+/358^-$)			
23.5 min	C^- (301^-)	C^- (301^-)	C^- (301^-)	C^- (301^-)				
23.8 min						J^{+-} ($611^+/609^-$)	J^{+-} ($611^+/609^-$)	E^+ (314^+) J^{+-} ($611^+/609^-$)
24.5 min	H^{+-} ($397^+/395^-$)	H^{+-} ($397^+/395^-$)	H^{+-} ($397^+/395^-$)	H^{+-} ($397^+/395^-$)				
25.0 min						G^+ (523^+)	G^+ (523^+)	F^+ (344^+)
28.3 min						Quercitrin$^+$ ($449^+/303^+$)	Quercitrin$^+$ ($449^+/303^+$)	Quercitrin$^+$ ($449^+/303^+$)
28.5 min				L^+ ($433^+/271^+$)				
31.1 min						Kaempferol-3-O-L-rhamnosyl$^+$ ($433^+/287^+$)	Kaempferol-3-O-L-rhamnosyl$^+$ ($433^+/287^+$)	Kaempferol-3-O-L-rhamnosyl$^+$ ($433^+/287^+$)
31.5 min			D^+ (373^+)					
(B) Zorbax column								
3.5 min				Mallonanoside B^{+-} ($333^+/331^-$)		Mallonanoside B^{+-} ($333^+/331^-$)	Mallonanoside B^{+-} ($333^+/331^-$)	Mallonanoside B^{+-} ($333^+/331^-$)
3.8 min				Mallonanoside A^{+-} ($347^+/345^-$)		Mallonanoside A^{+-} ($347^+/345^-$)	Mallonanoside A^{+-} ($347^+/345^-$)	Mallonanoside A^{+-} ($347^+/345^-$)
10.1 min								E^+ (314^+)
10.7 min	A^- (633^-)	A^- (633^-)	A^- (633^-)	A^- (633^-)				
	B^- (463^-)	B^- (463^-)	B^- (463^-)	B^- (463^-)				
13.1 min	C^- (301^-)	C^- (301^-)	C^- (301^-)	C^- (301^-)				
13.2 min	K^- (305^-)	K^- (305^-)	K^- (305^-)					
13.4 min					I^{+-} ($360^+/358^-$)			
14.1 min	H^{+-} ($397^+/395^-$)	H^{+-} ($397^+/395^-$)	H^{+-} ($397^+/395^-$)	H^{+-} ($397^+/395^-$)				
14.3 min			D^+ (373^+)	L^+ ($433^+/271^+$)				
15.1 min						J^{+-} ($611^+/609^-$)	J^{+-} ($611^+/609^-$)	J^{+-} ($611^+/609^-$)
17.1 min						Quercitrin$^+$ ($449^+/303^+$)	Quercitrin$^+$ ($449^+/303^+$)	Quercitrin$^+$ ($449^+/303^+$)
19.2 min						Kaempferol-3-O-L-rhamnosyl$^+$ ($433^+/287^+$)	Kaempferol-3-O-L-rhamnosyl$^+$ ($433^+/287^+$)	Kaempferol-3-O-L-rhamnosyl$^+$ ($433^+/287^+$)

The compounds mallonanoside A, mallonanoside B, quercitrin and kaempferol-3-O-L-rhamnosyl were identified on both columns. On the Zorbax column, mallonanoside A and mallonanoside B were partly co-eluting in samples 27, 35, 36 and 37, revealing no information of their individual contribution to the antixodant activity. However, on the monolithic column both compounds were separated and their respective peaks corresponded to negative regression coefficients. Furthermore, the interpretation of the regression coefficients for the flavonoids quercitrin and kaempferol-3-O-L-rhamnosyl is more straightforward on the Zorbax column as clearer negative regression coefficients are obtained.

For the unidentified compounds, on the Zorbax column, compounds C and K were co-eluting in samples 16, 17 and 19, compounds H and D in sample 19 and compounds C, H, and L in sample 27. As the regression coefficients of these peak were negative, at least one of the underlying substances is potentially antioxidant. Complementary information on these compounds could be extracted from the results of the monolithic column, separating compounds C, D, H, K and L. When evaluating their regression peaks, only the chromatographic peaks of compounds C and H corresponded to negative regression peaks while the other compounds did not possess antioxidant acitivity according to the O-PLS model. Vice versa, the co-eluting compounds E and J (sample 37) on the monolithic column are separated on the Zorbax column where only compound E corresponds to a negative regression peak. No additional information regarding compounds A and B was revealed when comparing both columns. Compounds F and G were not detected on the Zorbax column.

In conclusion, the dissimilar chromatographic systems provided complementary information. Several compounds co-eluting on one system were separated on the dissimilar system and their corresponding regression peaks revealed additional information on the contribution of these compounds to the antioxidant activity of the samples. Future work may include the structural elucidation and purification of the indicated unidentified compounds A, B, C, E and H, as well as an examination of their individual antioxidant activity.

8. Conclusions

Fingerprints of 39 different *Mallotus* samples, differing in species, cultivation conditions, harvest season and/or part of the plant, were developed on two dissimilar chromatographic systems. Prior to chemometric treatment of the data it was chosen not to align the fingerprints as no LC–MS spectra of all samples were available and a proper alignment of such complex and diverse biological samples is far from evident using only the UV chromatographic profiles.

In a first step, an exploratory analysis using principal component analysis was performed on both data sets individually. The monolithic column data clustered the unknown sample 4 as *Mallotus paniculatus* and unknown samples 38 and 39 as *Mallotus apelta*. Analysis of the Zorbax data confirmed the classification of sample 4. The clustering tendency on the PCA score plots is based on the similarity of the fingerprint profiles on a given system. Similar samples result in similar fingerprints from each chromatographic system individually. As a consequence, the complementary information in the PC plots is limited.

Furthermore, the antioxidant activities of the samples were determined and modelled as a function of the fingerprints using the orthogonal projections to latent structures technique. The data from both chromatographic systems were modelled separately. The peaks potentially responsible for the antioxidant activity of the samples were indicated studying the regression coefficients of the models. LC–MS analyses of the indicated peaks of the eight highly active antioxidant samples revealed four known compounds (mallonanoside A, mallonanoside B, quercitrin and kaempferol-3-0-L-rhamnosyl) and 11 unknown compounds. On both systems, several indicated peaks contained more than one compound co-eluting, but were separated on the dissimilar column. As a negative regression peak for co-eluting compounds reveals no useful information on the contribution of the individual compounds, one must take into account the regression peaks of the separated compounds on the dissimilar system.

The monolithic column separated mallonanoside A and mallonanoside B, partly co-eluting on the Zorbax column. Compounds C and K, H and D and C, K and L were also co-eluting on the Zorbax column and separated on the monolithic. Vice versa, the Zorbax column separated compounds E and J, which were co-eluting on the monolithic system. Combining the information from both systems indicated compounds C, E and H as potentially antioxidant and compounds D, J, K and L as non-antioxidant. Compounds A and B were co-eluting and indicated as potentially antioxidant on both systems.

Acknowledgements

Bieke Dejaegher is a post-doctoral fellow of the Fund for Scientific Research (FWO), Flanders, Belgium.

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