Development of HPLC fingerprints for *Mallotus* species extracts and evaluation of the peaks responsible for their antioxidant activity

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ABSTRACT

Some *Mallotus* species are used in traditional medicine in Vietnam. To use certain species in Western medicines or as food supplements, they should be identified and quality control should be more strict, for instance, to avoid the erroneous switching of species. In species with interesting activities, the compounds responsible for them should be identified. For these identifications, HPLC fingerprint methodology can be used.

In this paper, HPLC fingerprints of different lengths were developed for a number of *Mallotus* species. Secondly, a multivariate regression model was constructed to model the antioxidant activity of the *Mallotus* samples from the HPLC fingerprints with the aim to indicate peaks possibly responsible for this activity. For this purpose, after data pretreatment, the calibration technique partial least squares (PLS) was applied.

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1. Introduction

The *Mallotus* genus, belonging to the Euphorbiaceae family, is widely distributed in Vietnam and the south of China. In Vietnam, roots, stem barks, leaves and fruits of *Mallotus* species are for hundreds of years used in traditional medicine for the treatment of chronic hepatitis and enteritis [1]. According to the World Health Organization (WHO) [2,3], the use of herbal medicines is gaining importance, even in Western medicines. A major problem is that the quality of the herbs is not standardized. To avoid side/interaction effects or the exchanges between herbs, it is extremely important only to use medicines of which the quality is controlled. Until now *Mallotus* traditional medicines are not officially recognized worldwide because the quality, safety and efficacy are far from sufficient to meet the criteria set by the authorities.

Several *Mallotus* species exist, which often exhibit different pharmaceutical activities. In previous studies about the chemical components and pharmaceutical activities of *Mallotus* species, many compounds were isolated and pharmacologically active constituents were determined [4–12]. This was, for instance, the case with *Mallotus apelta*, *M. peltatus*, *M. japonicus*, and *M. roxburghianus*. Cytotoxic activity (*M. apelta*), antimicrobial activity (*M. peltatus*),

anti-inflammatory activity (*M. peltatus*, *M. japonicus*), and antioxidant activity (*M. japonicus*, *M. roxburghianus*) have been reported for given *Mallotus* species.

However, in any herbal medicine and its extracts, there are tens of unknown components, which are often only present in low amounts. Moreover, usually variability exists within the same herbal material [13,14]. The chemical components may vary depending on harvest seasons, plants origins, drying processes and other factors [15], and it is difficult to determine and isolate the whole of phytochemical constituents of herbal medicines [13,14,16]. Above all, identifying only one or some compounds hardly describes the complex extracts, and will therefore not be reliable enough for the quality control of these extracts. Moreover, extracts of the different species from a given genus, e.g. *Mallotus*, can have a very different composition.

Recently, the chromatographic fingerprint technique was introduced as a tool to evaluate the quality of herbal samples or their derived products [16–23]. This technique has been accepted by the WHO as a strategy for the quality assessment of herbal medicines [2,3] and recently gained more and more attention because it emphases on the characterization of the complete sample composition.

However, extracting information from a chromatographic fingerprint with many peaks is not evident. The applied methods will depend on the goal of the fingerprint analysis. Fingerprints might be used for identification purposes, e.g. to identify, for instance,

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extracts sold as coming from a given plant, but in fact originating from another. Other applications can be the identification of the geographical origin of given plant samples, or the modeling of a given activity.

The determination of standard compounds as markers in order to understand bioactivities and possible side effects of active compounds of the herbal medicines, and to enhance the product quality, also occasionally is applied [24]. However, this kind of determination does not give a complete picture of a herbal product, because multiple constituents are usually responsible for its therapeutic effect(s). These multiple constituents may work synergistically and could hardly be separated into active parts.

Nowadays, the combination of (hyphenated) chromatographic instruments and chemometrical approaches for data (pre-)treatment allows a fast investigation of the herbal samples [21,25–35].

The goal of this paper is to develop chromatographic fingerprints of a series of *Mallotus* samples from different species, origins, and/or collection times, and to indicate from them the compounds/peaks responsible for the antioxidant activity of given species. For some species, samples from different Vietnamese regions were collected, allowing to evaluate similarities and differences in chemical composition and activity.

HPLC fingerprints of different lengths (60, 35, and 22.5 min) were developed for 26 *Mallotus* extracts from 17 identified and 4 unidentified species. For all extracts, the antioxidant activity was determined with a 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity test. These activities were measured in two different laboratories, according to the methods described in [36,37] and in [38], respectively. Secondly, it was tried to link the observed antioxidant activity to given peaks in the HPLC fingerprints.

2. Theory

2.1. Data preprocessing

The results of a chemometric technique are influenced by the applied data preprocessing. In this paper, different methods to pretreat the data set, i.e. column centering, normalization and standard normal variate (SNV), were evaluated [27,39].

Column centering removes the column mean from each corresponding column. Normalization of the chromatograms scales the rows to a constant total. In our situation, normalization was performed by dividing each row by its corresponding norm. The standard normal variate (SNV) transformation corresponds to row centering, followed by row scaling, where row centering removes the row mean from each corresponding row and row scaling divides each row by its corresponding row standard deviation.

2.2. Exploratory analysis: principal component analysis

Principal component analysis (PCA) is applied for data compression and visualization. PCA produces so-called latent variables, here called principal components (PC's), which are linear combinations of the original manifest variables. The orthogonal PC's are constructed in such way that they maximize the description of the data variance in the $n \times p$ data matrix **X**. The projections of the objects (*Mallotus* samples) on PC_i are the scores on PC_i, and the projections of the variables (time points here) on PC_i are the loadings on PC_i. Thus, score plots give information related to the (dis)similarity of the objects, while on loading plots information about the contribution of the original variables to a given PC_i can be found. For more detailed information about PCA, we refer to [39].

2.3. Multivariate calibration: partial least squares

Partial least squares (PLS) [39–41] expresses the relation between the $n \times p$ data matrix **X** and a dependent $n \times 1$ vector **y** as follows:

$$\mathbf{y} = \mathbf{Xb} + \boldsymbol{\varepsilon} \tag{1}$$

where **b** is the $p \times 1$ vector of PLS regression coefficients, and $\boldsymbol{\varepsilon}$ the $n \times 1$ residual vector of **y**. PLS maximizes the covariance between **X** and **y**, and the model can be written as follows:

$$\mathbf{X} = \mathbf{TP^T} + \mathbf{E} \tag{2}$$

$$\mathbf{y} = \mathbf{TP^Tb} + \boldsymbol{\varepsilon} = \mathbf{Tq} + \boldsymbol{\varepsilon} \tag{3}$$

where **T** is the $n \times n$ score matrix, **P** the $p \times n$ loading matrix representing the regression coefficients of **X** on **T**, **E** the $n \times p$ residual matrix of **X**, and **q** the $n \times 1$ loading vector representing the regression coefficients of **y** on **T**. The regression coefficients **b** can be used to evaluate the contribution of the original variables to the (final) model [39–41].

To predict the response for a new chromatogram, represented by a $1 \times p$ row vector $\mathbf{x_i}$, the following equation can be applied

$$\hat{y}_i = \bar{y} + \mathbf{x_i}\mathbf{b} = \bar{y} + \mathbf{x_i}\mathbf{Pq} \tag{4}$$

where $\hat{y}_i$ is the predicted response value, and $\bar{y}$ the mean response value of all samples, used to build the model.

The optimal model complexity is determined by a leave-one-out cross-validation (LOO-CV) procedure. The root mean squared error of cross-validation (RMSECV) (Eq. (5)) is then calculated for PLS models with different complexities, i.e. with different numbers of PLS components

$$\mathrm{RMSECV}(f) = \sqrt{\sum_{i=1}^{N} \frac{(\hat{y}_{cv,i} - y_i)^2}{N}} \tag{5}$$

In Eq. (5), f is the number of PLS components, N the number of calibration samples, y_i the measured response of the ith sample, and $\hat{y}_{cv,i}$ the corresponding response predicted from the calibration model obtained without the ith sample. The RMSECV is then plotted as a function of the number of PLS components. The optimal complexity usually corresponds to that number leading to the (nearly) lowest RMSECV.

Afterwards, a PLS model can be validated with an independent test set. The root mean squared error of prediction (RMSEP) is then calculated for the elements of the test set [39]. However, in our situation, no division of the data into model and test set was made, since only 26 herbal samples were measured (rather small data set), and since prediction of the antioxidant activity for new samples was not our first concern. The indication of peaks responsible for this activity was. Moreover, the actual data set is not very well suited to build predictive models, since only few samples exhibited an antioxidant activity and a majority did not.

3. Experimental

3.1. Herbs and preparation of the herbal extracts

26 *Mallotus* samples, belonging to 17 identified and 4 unidentified species, were collected in different Vietnamese regions (Table 1). To protect the forests, only the leaves were collected. The samples were authenticated by Professor Nguyen Nghia Thin (Hanoi National University, Vietnam). For some species, samples were collected in different provinces of Vietnam and/or at different collection times, in order to evaluate similarities and differences in chemical composition and antioxidant activity.

Table 1
Mallotus samples: species, origin, collection time, voucher number, and the DPPH scavenging activity results, i.e. (a) %SA results, and (b) %DPPH results. (c) Predicted %DPPH values using the PLS model, built with the DPPH results from (b) as response and the 22.5 min HPLC fingerprint signals as independent variables, after normalization and column centering.

Sample	Species	Origin	Collection time	Voucher number	(a) Average %SA	s	(b) Average %DPPH	s	(c) Predicted %DPPH
1	*Mallotus luchenensis*	Son La	July 2006	01	3.0	2.0	82.0	12.1	78.2
2	*Mallotus microcarpus*	Son La	July 2006	02	7.7	1.8	63.6	13.0	67.7
3	*Mallotus barbatus*	Son La	July 2006	03	86.1	1.1	79.4	9.7	74.7
4	*Mallotus* sp1	Van Hoa	April 2006	MA07	3.4	1.1	113.0	20.5	90.9
5	*Mallotus barbatus*	Hagiang	November 2006	NT01	34.1	1.2	77.2	10.4	80.0
6	*Mallotus paniculatus*	Hagiang	November 2006	NT02	35.1	1.3	82.2	5.5	83.3
7	*Mallotus metcalfianus*	Hagiang	November 2006	NT03	75.7	1.3	51.1	14.6	73.2
8	*Mallotus apelta*	Tam Dao	July 2006	MA01	25.0	1.0	94.5	0.4	94.8
9	*Mallotus apelta*	Tam Dao	December 2006	MA02	27.5	1.3	92.5	3.3	99.3
10	*Mallotus paniculatus*	Tam Dao	April 2006	MA03	46.5	2.2	58.4	5.4	52.1
11	*Mallotus* sp2	Langson	March 2006	SP4	67.9	3.0	56.8	3.9	69.1
12	*Mallotus philippinensis*	Langson	March 2006	SP5	59.1	3.2	98.9	12.7	70.4
13	*Mallotus macrostachyus*	Langson	March 2006	MA11	28.5	2.1	75.7	2.2	69.0
14	*Mallotus microcarpus*	Quangbinh	March 2006	MA12	10.1	2.1	83.1	2.0	87.5
15	*Mallotus pallidus*	Quangbinh	March 2006	MA13	18.6	2.3	65.3	1.9	53.6
16	*Mallotus oblongifolius*	Quangtri	March 2006	MA14	90.7	1.1	6.7	0.3	5.4
17	*Mallotus floribundus*	Langson	November 2006	MA15	91.7	2.1	6.4	0.2	24.1
18	*Mallotus cuneatus*	Langson	November 2006	MA16	14.8	2.2	86.9	3.2	90.3
19	*Mallotus cuneatus*	Quangbinh	December 2006	MA17	94.6	2.2	10.3	4.1	44.2
20	*Mallotus* sp3	Quang tri	December 2006	MA18	−2.3	1.2	91.6	4.1	99.1
21	*Mallotus yunnanensis*	Lang Son	November 2006	MA19	4.6	1.3	91.6	6.6	83.2
22	*Mallotus poilanei*	Ke Bang	March 2006	MA20	0.7	3.1	90.5	7.0	92.4
23	*Mallotus hookerianus*	Dakrong	March 2006	MA22	92.1	2.1	50.0	4.6	45.8
24	*Mallotus nanus*	Daclak	March 2006	MA23	84.0	0.1	78.4	9.5	68.8
25	*Mallotus* sp4	Daclak	March 2006	MA24	24.6	1.0	56.9	11.7	61.8
26	*Mallotus oreophilus*	LaoCai	June 2006	M25	9.1	1.8	88.8	10.5	91.8

To prepare the herbal extract, 2.5 g plant sample (leaves) was weighed and extracted three times with 25 mL methanol in an ultrasonic bath (Branson Ultrasonic Corporation, Connecticut, USA), each time at a temperature between 40 and 50 °C during 60 min. Then the combined extracts were filtered through a 240 nm pore size filter paper (Whatman, Hanoi, Vietnam) and evaporated at decreased pressure (60 Pa) at a temperature of 50 °C. This crude extract was split into three parts, i.e. one was used for the DPPH radical scavenging assay, one for the HPLC analysis, and the last was kept as a library sample for reference purposes.

3.2. DPPH radical scavenging assay

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity test can be performed in different ways. Once the method described in [36,37] was used, and once that in [38]. The absorption of visible light by DPPH disappears when DPPH is reduced, i.e. in the presence of an antioxidant. The antioxidant activity can be expressed either as lost DPPH absorbance [36,37] or remaining DPPH concentration [38].

According to [36,37], the free radical scavenging activity (%SA) was evaluated by the decrease in the colorimetric absorbance of DPPH, and expressed as follows:

$$\%SA = 100 \times \left(\frac{A_{control} - A_{sample}}{A_{control}} \right) \tag{6}$$

where $A_{control}$ and A_{sample} are the absorbances at $\lambda = 517$ nm of the control (solvent dimethylsulfoxide (DMSO)) and the sample (extract dissolved in DMSO), respectively, after 30 min reaction time. The larger %SA of a sample is, the higher its antioxidant activity. The reported results (Table 1) were average values of three measurements, average %SA, and their standard deviations, s. The mean standard deviation for the 26 *Mallotus* samples was 1.7. However, it needs to be mentioned that this error only represents the repeatability of the absorbance measurement, and not that of the whole method. The method repeatability is higher, as can be seen further.

According to [38], the free radical scavenging activity was expressed as the percentage remaining DPPH concentration as follows:

$$\%DPPH_{remaining} = 100 \times \frac{[DPPH_{20}]}{[DPPH_0]} \tag{7}$$

where $[DPPH_{20}]$ and $[DPPH_0]$ are the DPPH concentrations of the sample (extract dissolved in methanol) and the control (solvent methanol), respectively, after 20 min reaction time. Measurements are performed at $\lambda = 515$ nm, and all DPPH concentrations were estimated relative to a linear calibration line, i.e. 1.0, 2.5, 5.0, 10.0, 25.0 and 50.0 µg/mL DPPH in methanol. Contrary to the former approach, in this situation, the smaller %DPPH$_{remaining}$ of a sample is, the higher its antioxidant activity. The reported results (Table 1) were average values of three independent measurements, average %DPPH, and their standard deviations, s. Here, the mean standard deviation, which represents method repeatability, was found to be 6.9.

3.3. Development of HPLC fingerprints

3.3.1. Equipment, chemicals and reagents

An Agilent 1050 HPLC system (Waldbronn, Germany), consisting of a vacuum degasser, quaternary pump, autosampler and a variable wavelength UV detector, was used. Chemstation for LC (Agilent) acquired and processed the analytical data.

For the chromatographic analyses, either two coupled Chromolith™ Performance RP-18e columns (100 mm × 4.6 mm I.D.) with a Chromolith guard column RP-18e (5 mm × 4.6 mm I.D.), or a Lichrospher 100 RP-18 column (125 mm × 4 mm I.D., 5 µm) with a guard column (5 mm × 4.6 mm I.D.), were used. All columns and guard columns are from Merck (Darmstadt, Germany).

HPLC grade methanol, acetonitrile (both from Fisher Scientific, Leicestershire, UK), trifluoroacetic acid (TFA) (Sigma–Aldrich, Steinheim, Germany), and MilliQ water (obtained from a MilliQ water
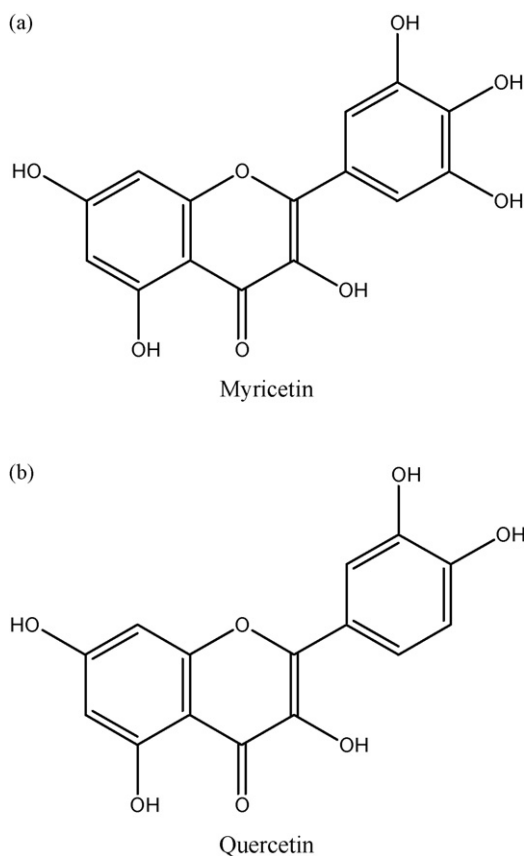
Fig. 1. Chemical structures of two flavonoids, (a) myricetin and (b) quercetin.

purification system, Millipore, Bedford, MA) were used to prepare the mobile phases. Besides with the applied online degassing system, all solvents were degassed prior to HPLC analysis on an ultrasonic bath (Branson Ultrasonic Corporation) during 15 min.

Injected standard compounds (Fig. 1) were myricetin (Sigma, Steinheim, Germany) and quercetin (Merck). Both compounds are flavonoids, which are known to possess antioxidant activity. They were injected to verify their presence or absence in given *Mallotus* samples.

3.3.2. Sample preparation for HPLC analysis

To prepare the samples for HPLC analysis, 50 mg crude extract was weighed and diluted to volume with methanol in a 2.0 mL volumetric flask. Then, the solution was mixed during 15 min on a shaking bath (Edmund Bühler shaking bath, Vel, Leuven, Belgium), and filtered through a filter of Schleicher & Schuell (Dassel, Germany) with a diameter of 125 mm and a pore size smaller than 2 µm. This was followed by filtration through a 25 mm syringe filter (VWR International, Leuven, Belgium) having a polypropylene membrane with 0.2 µm pore size.

To prepare the standard compound solutions, 1.0 mg standard was weighed, dissolved, and brought to volume with methanol in a 10.0 mL volumetric flask. Then, the same procedure as for the crude extracts, i.e. mixing and filtering, was followed.

3.3.3. Finally chosen chromatographic conditions

As stationary phase, the two coupled Chromolith™ Performance RP-18e columns (100 mm × 4.6 mm I.D.) with a Chromolith guard column RP-18e (5 mm × 4.6 mm I.D.) were selected. The mobile phase consisted of (A) 0.05% TFA in ACN, and (B) 0.05% TFA in MilliQ water. Gradient elution was applied. For the 60 min fingerprints, the gradient program was 5–20% A in 0–25 min, 20–95% A

in 25–50 min, and 95% A in 50–60 min interval. For the 35 min fingerprints, it was 5–25% A in 0–12.5 min, 25–95% A in 12.5–25 min, and 95% A in 25–35 min interval, and for the 22.5 min fingerprints 10–30% A in 0–6.25 min, 30–95% A in 6.25–12.5 min, and 95% A in 12.5–22.5 min interval.

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

3.4. LC–MS analyses

After the multivariate indication of the peaks potentially responsible for the antioxidant activity, the substances composing these peaks should be identified, isolated, and further examined. For this purpose, some preliminary LC–MS analyses were performed in order to obtain more information about the interesting components in the (highly) active antioxidant samples.

LC–MS analyses were performed in both positive and negative modes on three antioxidant samples: *Mallotus oblongifolius* (sample 16), *Mallotus floribundus* (sample 17), and *Mallotus cuneatus* (sample 19).

The LC–MS experiments were executed on an Alliance HPLC (Waters, Milford, Massachusetts, USA) equipped with an auto sampler and column oven. MS-detection was conducted using an ion trap LCQ-advantage system (Thermo Fisher Scientific, Waltham, Massachusetts, USA) equipped with an APCI interface. The mass spectrometer was operated in both the positive and negative ionization/detection modes. The following APCI inlet conditions were used. Nitrogen was used both as a nebulising gas and as a drying gas at a temperature of 450 °C. 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 the two modes 25 V of collision energy was applied. The system had a mass precision of 0.5 mass unit. Therefore, a difference of ±0.5 mass units between the value obtained and the real value is possible.

3.5. Data analysis

Computations were performed on a computer with an Intel 1.70 GHz Pentium-IV processor and 256 MB RAM, running Microsoft Windows XP and Matlab™ 6.5 (The MathWorks, Natick, MA). All data (pre)processing methods (column centering, normalizing, SNV, and PLS) are performed using m-files, written in Matlab version 6.5.

4. Results and discussion

4.1. DPPH radical scavenging assay

To compare both DPPH radical scavenging assays, the 100-%DPPH results are plotted as a function of the %SA results (Fig. 2). On the plot, the region where samples exhibit clear antioxidant activity, i.e. when both 100-%DPPH and %SA are larger than 50%, is indicated.

Fig. 2 shows that most *Mallotus* samples of the data set do not possess antioxidant activity. Only three *Mallotus* samples were considered to have a high antioxidant activity, i.e. *M. oblongifolius* (sample 16), *M. floribundus* (sample 17), and *M. cuneatus* (sample 19). *Mallotus hookerianus* (sample 23) and *Mallotus metcalfianus* (sample 7) were found to possess a moderate antioxidant effect. *Mallotus* sp2 (sample 11) is indicated as being antioxidant active from the %SA assay, and as borderline active from the %DPPH assay.

Sometimes both approaches lead to different conclusions concerning the antioxidant activity. For example, *Mallotus barbatus*
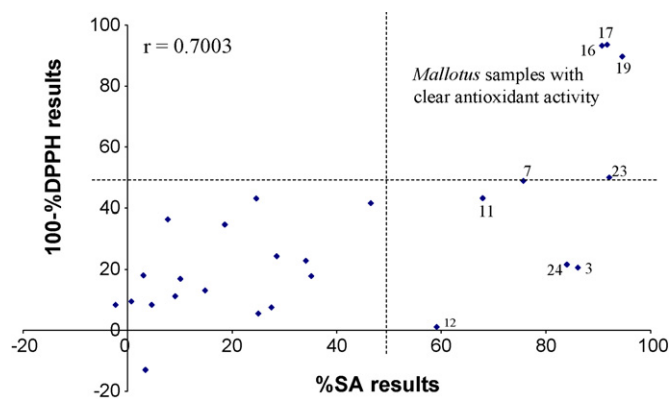
Fig. 2. 100-%DPPH results as a function of %SA results.

(sample 3), *Mallotus nanus* (sample 24), and *Mallotus philippinensis* (sample 12) have contradictory results, i.e. they are considered antioxidative from the %SA results, while from the %DPPH results, these samples are not considered antioxidative at all.

4.2. Development of HPLC fingerprints

4.2.1. Preliminary experimental work

Initially, the methodology proposed in [42] was followed. It consisted of a sample preparation procedure, followed by an experimental design approach to evaluate two chromatographic columns and two organic modifiers.

The herbal extract, chosen for the preliminary work, was the one possessing the highest antioxidant activity, according to the %SA results (Table 1), i.e. the extract of *M. cuneatus* (sample 19 in Table 1).

In the proposed sample preparation [42], 1 g herb is extracted in 20.0 mL 65% ethanol during 15 min on a shaking bath, followed by filtration. Because the actual samples were crude extracts, 50 mg was weighed and dissolved in either 2.0 mL MeOH (sample A) or 2.0 mL 65% ethanol (sample B), followed by mixing during 15 min on a shaking bath and filtration. Both preparations were injected under the four experimental conditions defined below.

Then, a 2^2 full factorial design was performed (Table 2). In four experiments, two factors were varied. The first was the chromatographic column (stationary phase) with as level (+1) two coupled Chromolith^{TM} Performance RP-18e columns (100 mm × 4.6 mm I.D.) with Chromolith guard column, and as level (−1) a Lichrospher 100 RP-18 column (125 mm × 4 mm I.D., 5 μm) with guard column. The second factor was the type of organic modifier in the mobile phase, with as level (+1) methanol and as level (−1) acetonitrile. The mobile phase consisted of (A) 0.05% TFA in organic modifier, and (B) 0.05% TFA in MilliQ water. The total run time was 60 min. The initial gradient elution program was 5–95% A in 0–50 min, followed by isocratic 95% A during 10 min. Further, the experimental conditions described in Section 3.3.3 were applied.

It was found that the sample preparation in methanol (sample A) was preferred, since more peaks were observed in the fingerprints. From the four design experiments, it was decided to use the coupled monolithic silica columns as stationary phase and acetonitrile

Table 2
2^2 full factorial design.

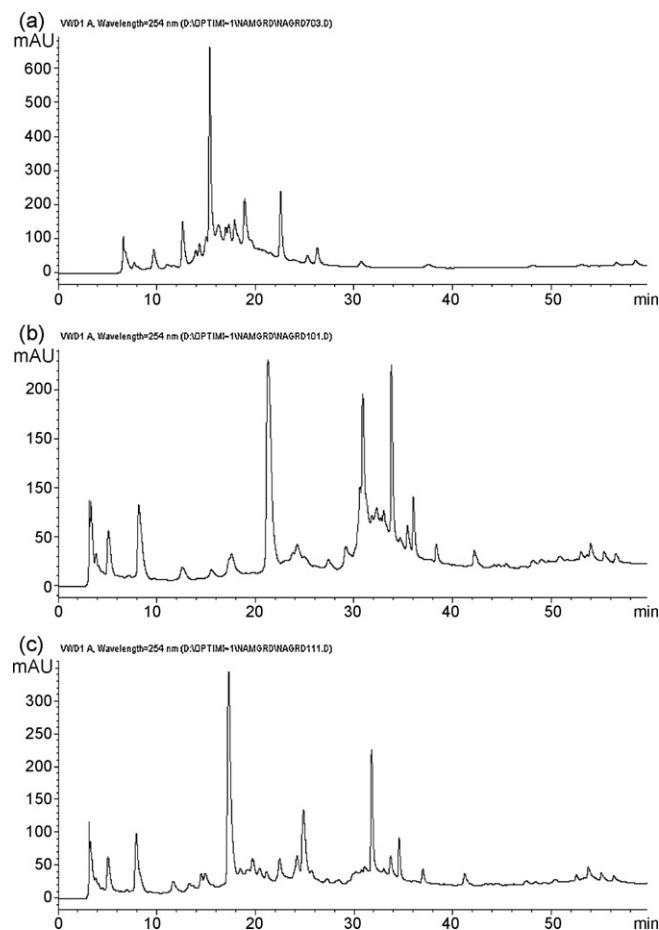| Experiment number	Factor 1: column	Factor 2: organic modifier
1	−1	−1
2	−1	+1
3	+1	−1
4	+1	+1

Fig. 3. Gradient optimization of the 60 min fingerprint of *Mallotus cuneatus* (sample 19 in Table 1). Applied gradients are (a) 5–95% A in 0-–50 min, and isocratic 95% A in 50–60 min; (b) 5–15% A in 0–25 min, 15–95% A in 25–50 min, and isocratic 95% A in 50–60 min; (c) 5–20% A in 0–25 min, 20–95% A in 25–50 min, and isocratic 95% A in 50–60 min.

as modifier for further development. These conditions led to the highest number of peaks observed and to a more stable baseline.

Further optimization was then performed, in which column length and gradient program were examined. Both two and three coupled monolithic columns were tested. The gradient described above was used, and the other conditions were as in Section 3.3.3. The pressure was exceeding the recommended values when coupling three columns, which may damage them. Additionally, no or only a small improvement in the fingerprints was obtained. Therefore, it was chosen to use the two coupled Chromolith^{TM} columns with a Chromolith guard column for further experiments.

Secondly, the gradient was adapted. All other conditions remained constant. The initial gradient results in the fingerprint, shown in Fig. 3a, where mainly peaks were seen from 6 to 30 min, and a considerable overlap occurred. Therefore the steepness of the gradient was adapted. A second gradient was 5–15% A in 0–25 min, 15–95% A in 25–50 min, and 95% A in 50–60 min. In the new fingerprint (Fig. 3b), peaks were now found from 3 to 60 min. However, there are still overlapping peaks in the region between 30 and 40 min. The third and finally selected gradient is described in Section 3.3.3, and the thus obtained fingerprint shown in Fig. 3c. The separation degree of the peaks was highest for the latter gradient.

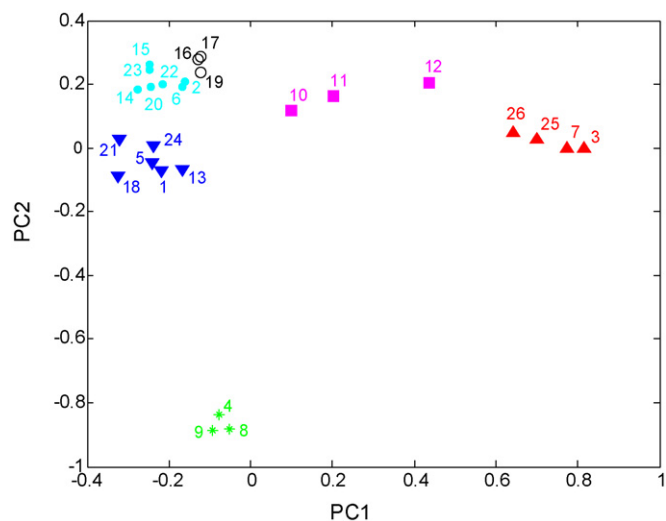### 4.2.2. Fingerprints of the Mallotus species

The experimental conditions for the 60 min HPLC fingerprints are described in Sections 3.3.2 and 3.3.3. The fingerprints obtained
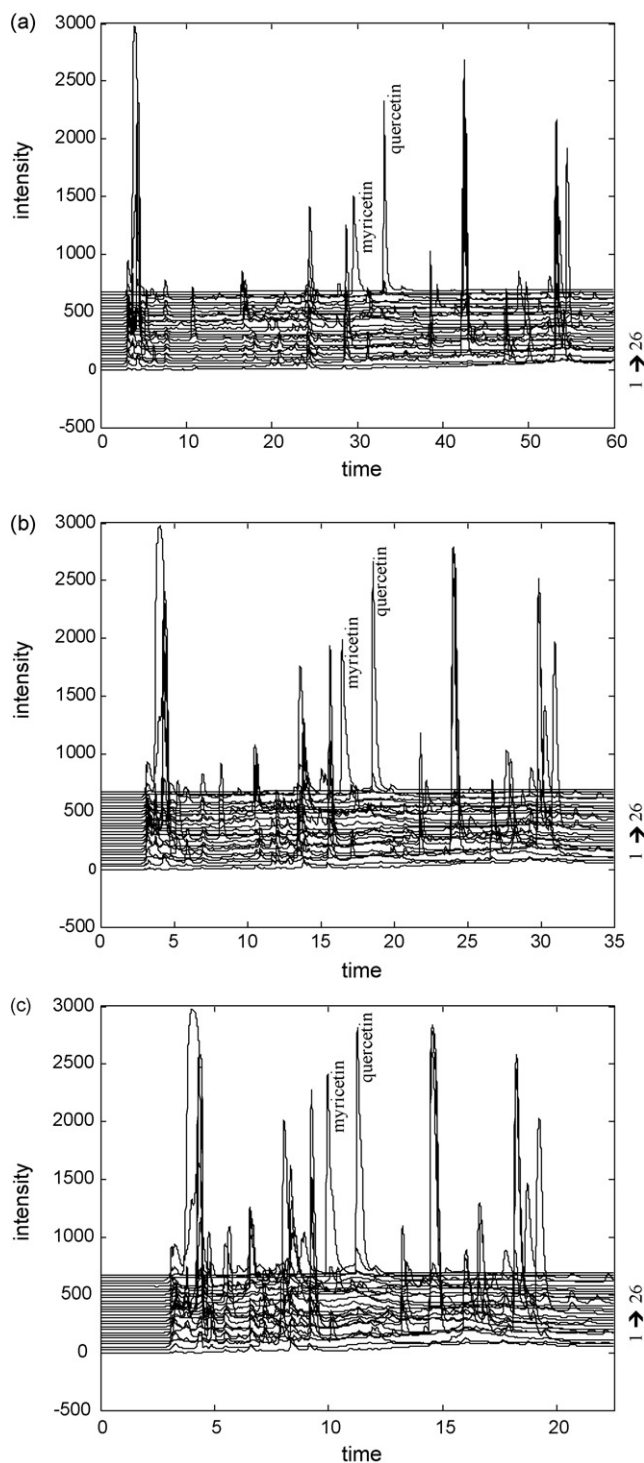
Fig. 4. Overlay of (a) 60 min, (b) 35 min, and (c) 22.5 min HPLC fingerprints for the 26 *Mallotus* samples (black solid lines, —) and the two standard compounds myricetin and quercetin (black dotted lines, - - -).

Fig. 5. PC1–PC2 score plot for the 26 *Mallotus* samples, using the 60 min HPLC fingerprint signals as independent variables and normalization followed by column centering as preprocessing. The following groups are distinguished: (a) samples 16-17-19 (black), (b) samples 3-7-25-26 (red), (c) samples 2-6-14-15-20-22-23 (cyano), (d) samples 10-11-12 (magenta), (e) samples 1-5-13-18-21-24 (blue), and (f) samples 4-8-9 (green). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

for the 26 *Mallotus* samples are shown in Fig. 4a. Since the 26 examined *Mallotus* samples at least belong to 17 different species (see Table 1), very divergent fingerprints were obtained (see also Section 4.3.2), probably because the chemical constituents differ considerably between the species. Therefore, possibly different pharmaceutical activities can be attributed to different species.

Since it is always desired to decrease analysis times, but maintaining the information, a protocol for 35 min fingerprints was also developed. When the peaks responsible for the antioxidant activity are still separated in the shorter fingerprints, this would lead to a decrease in analysis time and costs (e.g. less mobile phase consumption). The analysis time was reduced from 60 to 35 min by changing the gradient elution program. Initially, only the slope of the gradient was changed, resulting in 5–20% A in 0–12.5 min, 20–95% A in 12.5–25 min, and isocratic 95% A in 25–35 min. The gradient was then further optimized, similarly as for the 60 min fingerprints, and the finally chosen gradient is described in Section 3.3.3. The other experimental conditions were the same as for the 60 min fingerprints. The resulting 35 min fingerprints for the 26 *Mallotus* samples are presented in Fig. 4b. Although analysis times were reduced, similar profiles as in Fig. 4a are distinguished. However, some peaks, that were baseline separated in the 60 min fingerprints, are overlapping in the 35 min fingerprints.

Then it was tried to further reduce the analysis time to 22.5 min. This was again achieved by changing the gradient similarly as above, leading to 5–25% A in 0–6.25 min, 25–95% A in 6.25–12.5 min, and isocratic 95% A in 12.5–22.5 min. Again the gradient was further optimized, and the finally chosen gradient is described in Section 3.3.3. The other experimental conditions remained again the same as for the 60 and 35 min fingerprints. The 22.5 min fingerprints are shown in Fig. 4c. The major peaks can still be distinguished. However, as expected, more peaks are co-eluting or overlapping, compared to the longer fingerprints. Nevertheless, most peaks responsible for the antioxidant activity are still clearly visible and indicated when evaluating the regression coefficients (see further).

It is also observed that the two standards myricetin and quercetin were not or only in small amounts present in the samples. This implies that the antioxidant activity is probably due to other flavonoids or to tannins in the herbal extracts. These other compounds, responsible for the antioxidant activity, should thus be indicated on the fingerprints by evaluating the regression coefficients. In a later stage, these compounds should then be identified.

4.3. Data (pre-)treatment and multivariate data handling

4.3.1. Preprocessing

Prior to PCA analysis or to constructing a PLS calibration model, the fingerprint data was preprocessed. Different preprocessing
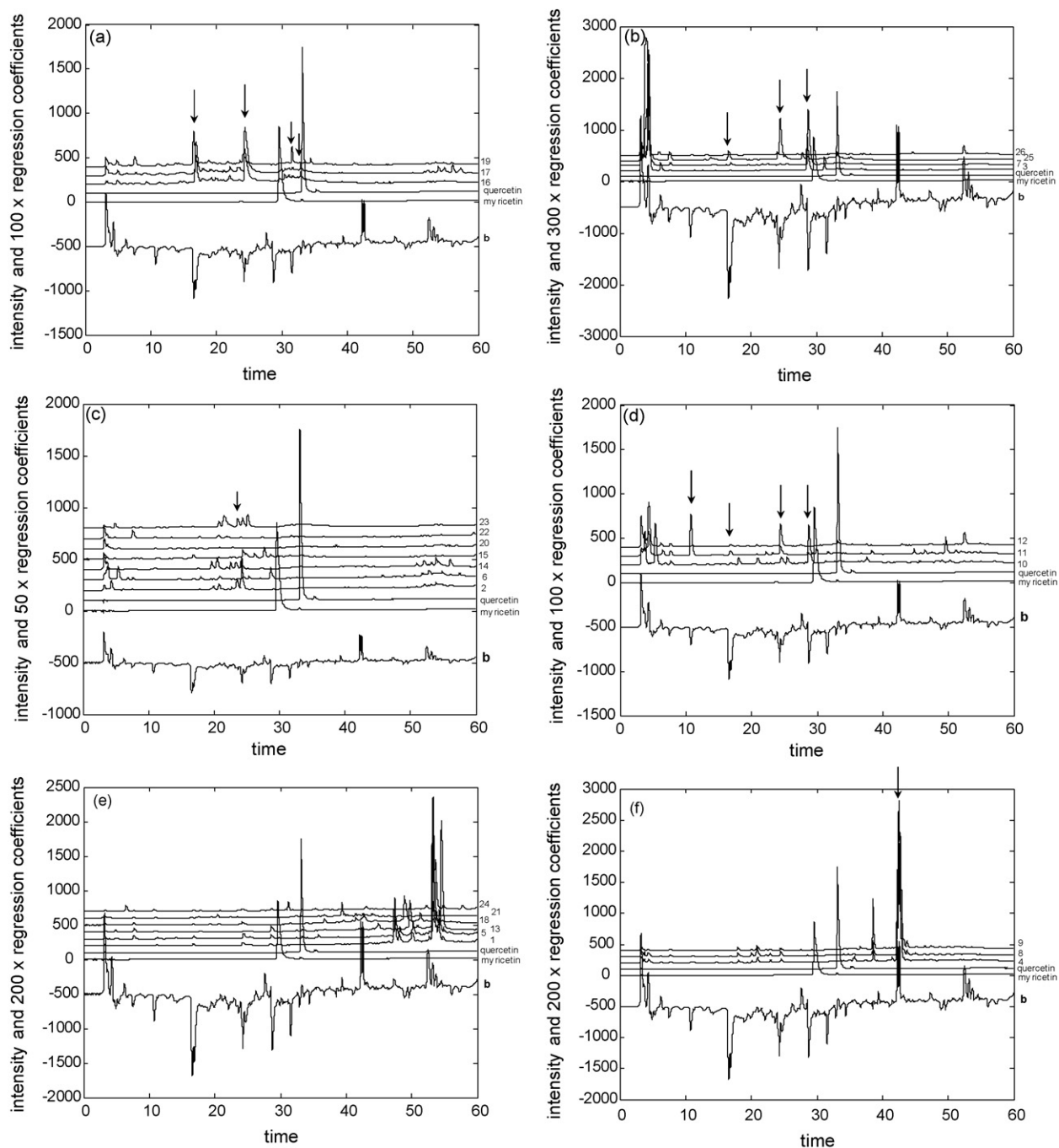
Fig. 6. The 60 min fingerprints, the PLS regression coefficients, and the chromatograms of myricetin (1st peak) and quercetin (2nd peak) (black dotted lines, ---). The fingerprints are grouped according to Fig. 5. For groups (a)–(d), major peaks with negative regression coefficients are indicated with an arrow (↓), while for group (f), peaks with positive regression coefficients are.

methods were evaluated, i.e. column centering, normalization followed by column centering, and standard normal variate (SNV) transformation followed by column centering.

Aligning the corresponding peaks is recommended, because shifts in retention time are observed between chromatograms (Fig. 4). For this purpose, a technique, called correlation optimized warping (COW) [43–45], was applied. However, the aligned data set lead to worse results than the non-aligned, probably because of the large diversity in species, which makes it not evident to align the chromatograms. Possibly peaks, considered as corresponding, match different components since the *Mallotus* species had very divergent fingerprints (see Fig. 4). For this particular data set, it is rather difficult to know which peaks correspond and should be

aligned, since no diode array detector (DAD) or mass spectrometry (MS) data for all samples was available. Thus, aligning peaks was not evident, because peaks representing different components can be forced to align. Therefore, in this study, it was decided to interpret the non-aligned chromatograms.

4.3.2. Principal component analysis

Principal component analysis (PCA) was applied on the three preprocessed data sets, to verify whether the extracts with high antioxidant activity could be distinguished on the score plots. However, regardless the applied preprocessing, no group of extracts with antioxidant activity could be clearly distinguished on any score plot.

For example, when applying normalization followed by column centering as preprocessing, i.e. the approach leading to the best predictive PLS models (see Section 4.3.3), the PC1–PC2 score plot, shown in Fig. 5, is obtained. Although the three samples with a high antioxidant activity (a), i.e. *M. oblongifolius* (sample 16), *M. floribundus* (sample 17), and *M. cuneatus* (sample 19), are situated together, they are not clearly separated from group(s) containing *Mallotus* samples without antioxidant activity.

Some groups can be distinguished on the PC1–PC2 score plot, i.e. (a) with samples 16-17-19, (b) with samples 3-7-25-26, (c) with samples 2-6-14-15-20-22-23, (d) with samples 10-11-12, (e) with samples 1-5-13-18-21-24, and (f) with samples 4-8-9. In Fig. 6, the fingerprints are presented per group. Each group contains similar fingerprints, and between groups the fingerprints are clearly different. Thus, mainly the variation caused by the differences in the fingerprints is revealed in the score plot. From the fingerprints of group (f), it can be concluded that *Mallotus* sp1 (sample 4) probably belongs to the species *M. apelta*, since exactly the same fingerprint profile as for the two *M. apelta* samples in this group is obtained. However, the goal of this paper is not identification or authentification of the samples by means of the fingerprints. The above assumption about *Mallotus* sp1 (sample 4) should be verified by a botanical authentication of the sample.

Since in Fig. 5, the three samples of group (f) are located far from all other groups along PC2, they were excluded from the data set **X**, and the PCA was repeated. From the thus obtained PC1–PC2 score plot (not shown), groups (b) and (d) are still clearly distinguished and separated from the other samples. The antioxidant samples of group (a) are now better separated from groups (c) and (e). However, an overlap occurred between the samples of groups (c) and (e).

4.3.3. Partial least squares

As mentioned in Section 2, since the total number of samples (26) is rather small and since prediction for new samples was not our first concern, no division was made into a calibration set to build a PLS model and a test set to validate the predictive properties. It also should be noticed that the actual data set is not so well suited to build models to predict the antioxidant activity, given the high fraction of non-active samples. However, our main concern is to focus on the indication of antioxidative peaks from the modeling results.

PLS models were built from the data matrix **X** containing the 26 *Mallotus* fingerprints and the response vector **y**, i.e. either the %SA or the %DPPH results. The optimal model complexity was determined from the RMSECV calculated, for models with different numbers of PLS components, by LOO-CV. In general, the simplest model with (nearly) the lowest RMSECV was chosen. Both the influences of the three preprocessing approaches and of the two series of antioxidant test results on the predictive ability of the PLS model were evaluated. The RMSECV's of the different PLS models are given in Table 3.

Generally, the RMSECV was smaller when using the %DPPH results. Possibly, the replicated %DPPH results are more representative for the antioxidant test than those of %SA (see higher) where the error of the method seems to be underestimated. Concerning the preprocessing methods, in general, normalization followed by column centering leads to PLS models with a (slightly) better predictive ability. For the different fingerprint lengths, the one of 22.5 min had the best results.

In Table 1, the predicted %DPPH values, obtained from the latter fingerprints when using normalization followed by column centering as preprocessing, are given for the 26 *Mallotus* samples. It can be concluded that some predictions are not very good (Fig. 7). The reason is to be found in the small number of (intermediate) antioxidative samples available, as already mentioned higher.
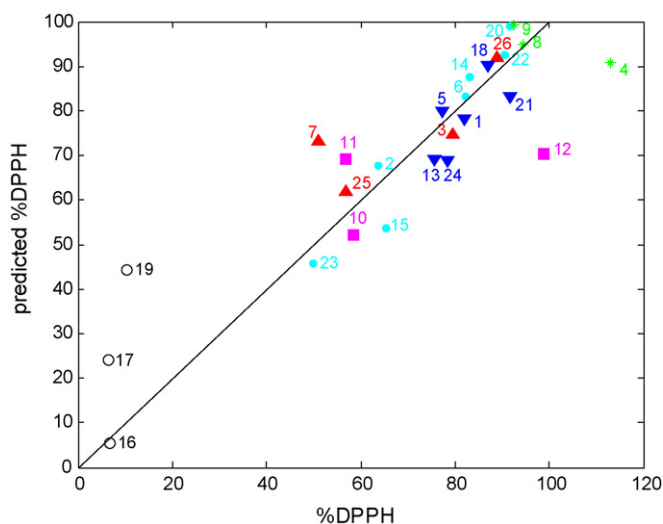
Table 3
Number of PLS components (#) and RMSECV for the PLS models, build based on the 60, 35, and 22.5 min fingerprints. Both the influence of the three preprocessing approaches and the two antioxidant tests on the predictive ability of the PLS model was evaluated.

Fingerprint length (min)	Preprocessing	%SA		%DPPH	
		#	RMSECV	#	RMSECV
60	Column centering	4	27.8	4	15.3
	Normalization and column centering	3	24.1	5	12.5
	SNV and column centering	4	23.7	3	13.9
35	Column centering	3	28.8	5	13.1
	Normalization and column centering	3	25.3	6	9.9
	SNV and column centering	2	25.8	7	12.0
22.5	Column centering	3	26.5	5	12.3
	Normalization and column centering	3	23.7	6	9.0
	SNV and column centering	3	24.0	4	11.7

To evaluate the contribution of the substances/peaks to the final PLS model, the regression coefficients are plotted. These coefficients (black lines, —) are added in Fig. 6, to the HPLC fingerprints. The peaks of the two standard compounds (dotted lines, ----), myricetin (1st peak) and quercetin (2nd peak), also were. Although the three antioxidant active samples (16, 17, 19) are not largely separated from the other samples on the PC1–PC2 score plot, a clear difference between the active and the non-active samples was noticed when comparing the fingerprints and the regression coefficients.

Peaks with major negative regression coefficients are indicated in Fig. 6a–d. They contribute largely to the PLS model [39–41]. They correspond to compounds with either antioxidant activity or to compounds for which the concentration has a similar behavior as the antioxidant activity. The concentration of the latter compounds increases with an increase of the antioxidant activity in the herbal extract, i.e. when %DPPH becomes smaller.

In Fig. 6a, the 60 min fingerprints of group (a), containing *M. oblongifolius* (sample 16), *M. floribundus* (sample 17), and *M. cuneatus* (sample 19), are plotted together with the regression coefficients. These samples have an antioxidant activity, and contain compounds (indicated with ↓) for which negative regression coefficients are observed. In Fig. 6b, the fingerprints of group (b) are

Fig. 7. Predicted %DPPH values as a function of the measured %DPPH values (see Table 1). The samples are labeled as in Fig. 5.

presented, which contains a sample with intermediate antioxidant activity (*M. metcalfianus* (sample 7)), while in Fig. 6c, those of group (c) are shown, which contains another sample with intermediate antioxidant activity (*M. hookerianus* (sample 23)). These two fingerprints also have some peaks with negative regression coefficients (see Fig. 6b and c), although those for sample 23 are quite small. When looking more closely at Table 1, five other samples could also be considered moderately active (50% ≤ %DPPH ≤ 65%), i.e. *Mallotus microcarpus* (sample 2), *Mallotus paniculatus* (sample 10), *Mallotus* sp2 (sample 11), *Mallotus pallidus* (sample 15), and *Mallotus* sp4 (sample 25). From Fig. 6b–d, it can be seen that their fingerprints also contain some peaks with negative regression coefficients.

In the following, the fingerprints of samples from the same species were compared. For both *M. apelta* samples (8, 9) (Fig. 6f), both *M. microcarpus* samples (2, 14) (Fig. 8a), and both *M. paniculatus* samples (6, 10) (Fig. 8b), similar fingerprints were obtained. Thus, these samples have a similar chemical composition. The two *M. apelta* samples (8, 9) showed similar antioxidant activity results, i.e. were non-active (Table 1). For the *M. microcarpus* samples (2, 14), differences in the antioxidant activity were observed. The compound causing a negative regression coefficient is present in a larger amount in sample 2 than in sample 14, possibly explaining why sample 2 has a lower %DPPH$_{remaining}$ (see Table 1 and Fig. 8a). A similar observation was made for the two *M. paniculatus* samples (6, 10), possibly explaining why sample 10 has a lower %DPPH$_{remaining}$ than sample 6 (see Table 1 and Fig. 8b).

However, for both *M. barbatus* samples (3, 5) (Fig. 8c) and both *M. cuneatus* samples (18, 19) (Fig. 8d), dissimilar fingerprints were obtained. This explains why these samples were not located in each others vicinity on the PC1–PC2 score plot. Their chemical composition is different. Possibly, this is caused by the different origin of the samples. Both *M. barbatus* samples (3, 5) were considered nonactive, while the *M. cuneatus* samples (18, 19) exhibited a different activity, i.e. sample 19 was highly active, while sample 18 was not (Table 1). In the fingerprints, the compounds with negative regression coefficients are present in sample 19, but hardly in sample 18 (Fig. 8d).

In a search for interesting and possibly new antioxidant compounds, in a next step, the substances from the relevant peaks should be further identified, isolated, and examined. Preliminary LC–MS experiments have been performed in both positive and negative modes on the three highly antioxidant active samples of group (a): *M. oblongifolius* (sample 16), *M. floribundus* (sample 17), and *M. cuneatus* (sample 19).

Most peaks were observed in the negative mode. In this mode, the three samples show common peaks at about 16.8 and 24.5 min. In the mass-spectra, three different components could be distinguished at 16.8 min, i.e. component A at 301 m/z [A−H]$^-$ and 414 m/z [A−H−TFA]$^-$, component B at 463 m/z [B−H]$^-$ and 576 m/z [B−H−TFA]$^-$, and component C at 633 m/z [C−H]$^-$ and 746 m/z [C−H−TFA]$^-$, with TFA equal to 113 amu (atomic mass unit). For these three samples, component A (301 m/z) could also be found at 24.5 min. In Fig. 9, the MS-spectra obtained at 16.8 and 24.5 min are presented for sample 17. Because of the difference of 162 amu in mass between components A and B, which corresponds to a hexose, component A at 24.5 min is most probably the eluting aglycon of component B, while component A found at 16.8 min is the aglycon formed by fragmentation of component B during MS analysis. Fur-
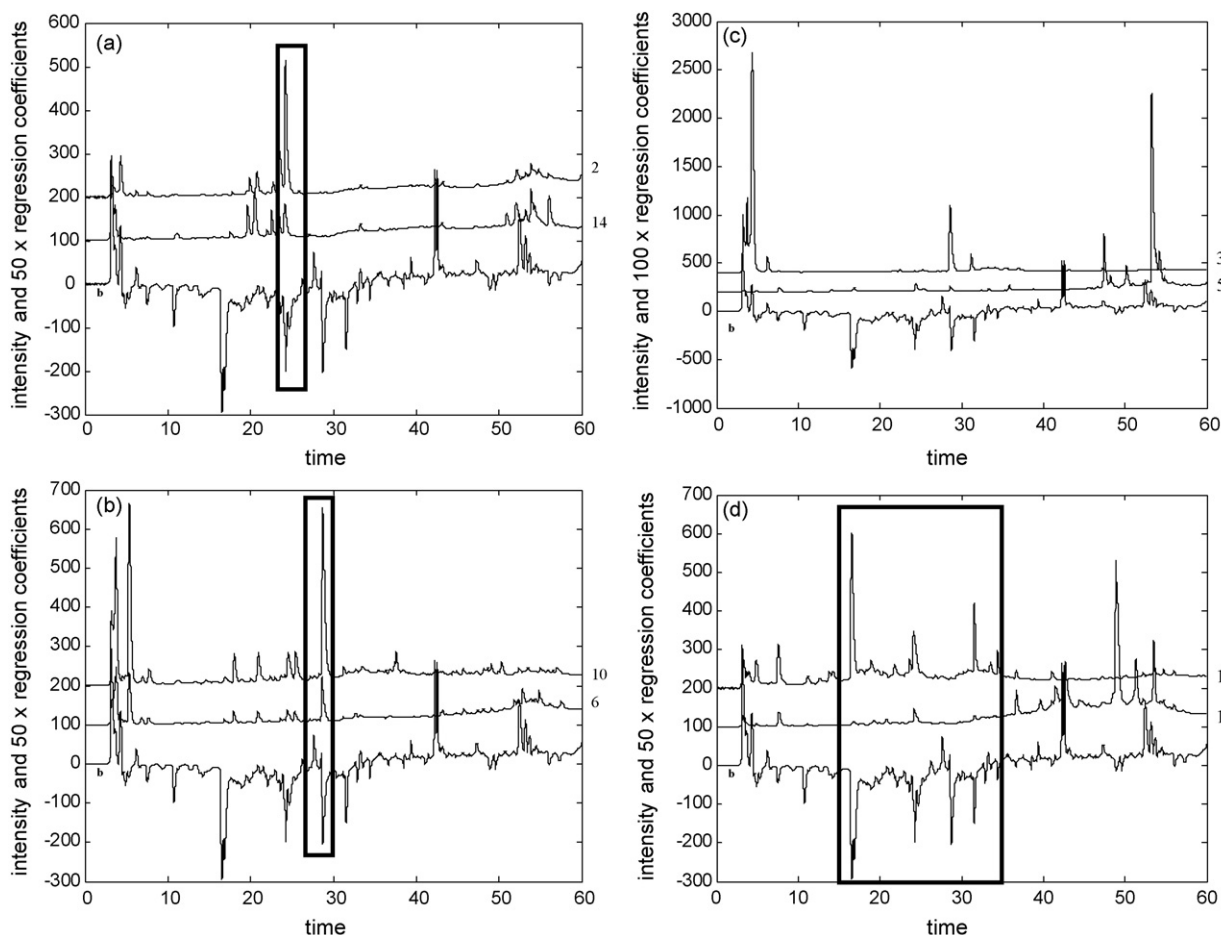

Fig. 8. The PLS regression coefficients plotted together with the 60 min fingerprints of (a) *Mallotus microcarpus* samples (2,14), (b) *Mallotus paniculatus* samples (6,10), (c) *Mallotus barbatus* samples (3,5), and (d) *Mallotus cuneatus* samples (18,19). Peaks with negative regression coefficients and occurring in different concentrations are indicated.
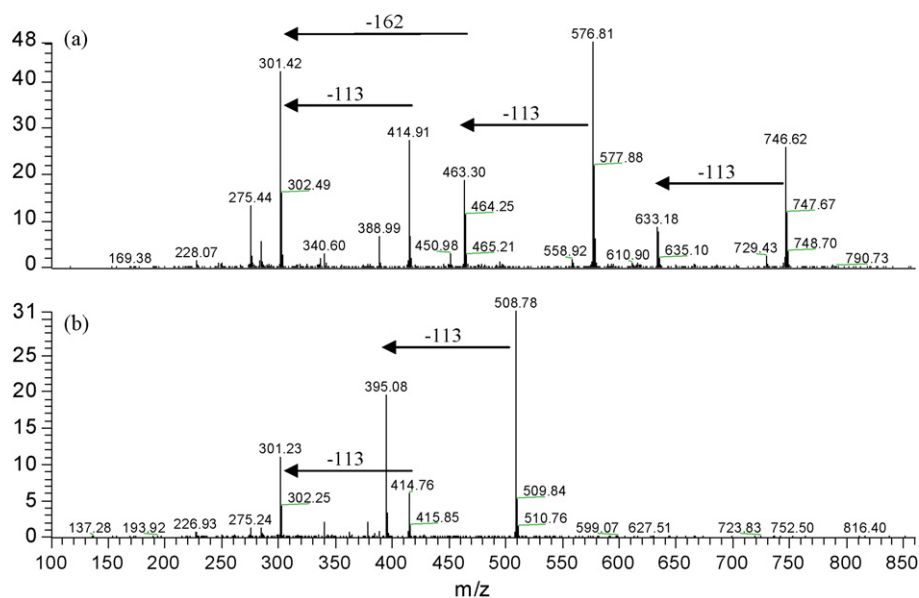
Fig. 9. MS-spectra obtained in negative mode for the peaks in sample 17 at (a) 16.8 and (b) 24.5 min.

thermore, sample 16 shows one extra component at about 24.5 min compared to the other two samples, i.e. component D at 211 m/z [D−H]⁻ and 324 m/z [D−H−TFA]⁻. Sample 17 shows an extra component at about 24.50 min compared to the other two samples, i.e. component E at 395 m/z [E−H]⁻ and 508 m/z [E−H−TFA]⁻.

In positive mode, only for samples 16 and 19 peaks were observed. For sample 16, a peak was detected in positive mode at about 24.5 min, containing components F at 414 m/z [F+H]⁺ and G at 397 m/z [G+H]⁺ (Fig. 10). Since component F has an odd mass (413), it is possible that a nitrogen atom (N) is present in the molecule. Component G possibly is a fragmentation product of component F, since successive losses of 17 amu, which may account for the loss of NH₃, 18 amu, corresponding to the loss of H₂O, and 15 amu, represent-

ing the loss of CH₃, were observed (Fig. 10). For sample 19, a peak was detected in positive mode at about 31.5 min, corresponding to component I at 373 m/z [I+H]⁺.

The compounds from the relevant peaks should be further isolated, purified and identified. Identification of compounds can, for example, be done by comparing the MS-spectra with those of standards, or by deducing the structure of the compounds by means of MS/MS or nuclear magnetic resonance (NMR) analysis. Nevertheless, this involves future work for analysts with the necessary required expertise.

On the other hand, peaks with positive regression coefficients (Fig. 6f) possibly correspond to compounds reducing antioxidant activity, or more general to compounds for which the
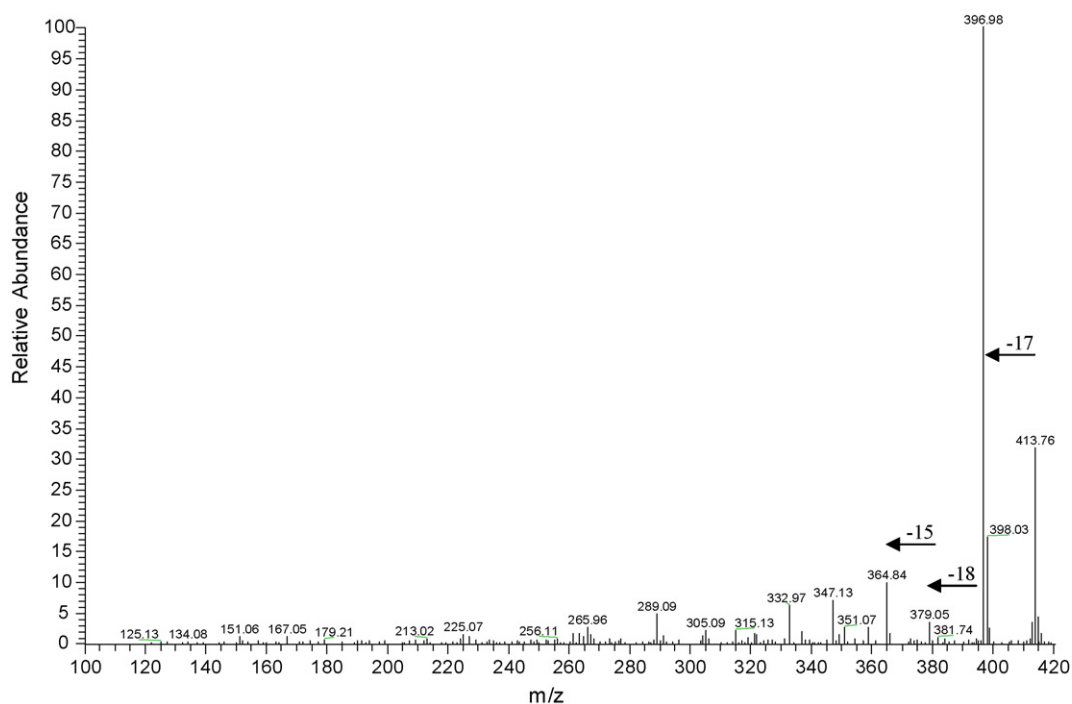

Fig. 10. MS-spectrum obtained in positive mode for the peak in sample 16 at 24.5 min.

concentration has an opposite behavior as the antioxidant activity. The concentration of these compounds increases with a decrease of the antioxidant activity in the herbal extract.

In Fig. 6f, the 60 min fingerprints for group (f), containing *Mallotus* sp1 (sample 4), *M. apelta* (sample 8), *M. apelta* (sample 9), are plotted. From earlier research, it is known that *M. apelta* has cytotoxic activity [8,10,11]. The peaks with positive regression coefficients are largely present in these three samples, possessing no antioxidant activity. Possibly, these peaks have cytotoxic activity, but this also needs further research.

5. Conclusions

In this study, HPLC fingerprints of *Mallotus* species were developed. A multivariate regression model was constructed to model the antioxidant activity of the *Mallotus* species samples from the HPLC fingerprints and to indicate peaks responsible for this activity.

The PLS model with the best predictive ability was obtained from the 22.5 min fingerprints. From comparing the peaks in the fingerprints with the regression coefficients of the model, peaks probably responsible for antioxidant activity can be indicated. In a next step, the substances from these relevant peaks should be identified, isolated, and further examined.

Acknowledgements

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