Talanta 83 (2011) 1188–1197

Contents lists available at ScienceDirect

Talanta

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

Pressurized capillary electrochromatography in a screening for possible antioxidant molecules in *Mallotus* fingerprints: Challenges, potentials and prospects

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ARTICLE INFO

Article history:
Available online 18 August 2010

Keywords:
Pressurized capillary electrochromatography
Antioxidant compounds
Fingerprints
PLS

ABSTRACT

Because of its eminent high resolution potential and minimal solvent consumption, pressurized capillary electrochromatography (pCEC) may offer an interesting alternative to HPLC for screening applications that need to resolve complex samples. In this paper, its potential was assessed in a screening of plant extracts from *Mallotus* species to indicate compounds with possible antioxidant activities by means of a PLS model built from their pCEC fingerprints. The main aim of this research was to find out whether pCEC can have an added value for this application. To get a complete overview of the techniques potential for this application, it was also assessed whether the technique can meet the requirements in terms of precision, sensitivity and column robustness. Encountered benefits and downsides were reported. Fingerprints with satisfactory sensitivity and precision could be obtained by concentrating the sample 5-fold and using optimized rinsing procedures, respectively. From the generated pCEC fingerprints of 39 *Mallotus* samples and their respective DPPH radical scavenging activity test results, a three-component PLS model was being built. The model proved good predictive abilities and easily allowed the indication of possible antioxidant compounds in the fingerprints. Despite its much higher peak capacity, the performance of pCEC to fingerprint the majority of the *Mallotus* extracts did not surpass that of a custom HPLC method. This was also reflected in its comparable power to indicate possible antioxidant compounds in the fingerprints after modeling. Because of its low detection sensitivity and modest column robustness, the benefit of the lower solvent consumption was partly paid-off by the current need for more system maintenance, also limiting the sample throughput. For the considered screening application, pCEC may suit as a viable but no preferred alternative technique.

1. Introduction

In the last decades, significant links between oxidative stress and some prevalent cancers and inflammatory diseases have been established [1,2]. As a result, antioxidants, having protective actions against cellular damage caused by oxidative stress [3], have become well-liked subjects of study in the biomedical research field [4,5]. Since oxidative stress is also known to be involved in the ageing process, antioxidants have turned out being popular constituents of cosmetics and food supplements as well.

This amplifying interest in antioxidants has aroused great curiosity to find novel antioxidant molecules. The diversity in existing plant genera and species makes plants major resources of various antioxidant molecules. Screening of plant extracts, using chromatography along with fast post-column (bio) chemical detection [6], or along with chemometrics [7], is reported for the indication of (novel) antioxidant molecules. In the latter case, not all, but only the indicated peaks, possibly corresponding to antioxidant compounds, can then be further isolated, identified (*e.g.* by mass spectrometry (MS) or nuclear magnetic resonance (NMR)) and further studied.

Plant extracts are challenging samples, because they contain numerous metabolites, being extremely complex mixtures of small molecules. These metabolites are the result of complex plant biochemistry. Hence, differences in plant species and cultivation (and harvest) places and conditions, to name some, can influence the metabolite profile of the plant. It is thus of outmost importance that the chromatographic separation technique enables reflecting

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doi:10.1016/j.talanta.2010.08.006

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

Sample	Voucher number	Species	Origin	Collection time	Part of plant	%DPPH$_{rem}$
1	01	*Mallotus luchenensis*	Son La	July 2006	Leaves	82.0
2	02	*Mallotus microcarpus*	Son La	July 2006	Leaves	63.6
3	03	*Mallotus barbatus*	Son La	July 2006	Leaves	79.4
4	MA07	*Mallotus sp1*	Van Hoa	April 2006	Leaves	100.0
5	NT01	*Mallotus barbatus*	Hagiang	November 2006	Leaves	77.2
6	NT02	*Mallotus paniculatus*	Hagiang	November 2006	Leaves	82.2
7	NT03	*Mallotus metcalfianus*	Hagiang	November 2006	Leaves	51.1
8	MA01	*Mallotus apelta (Ma1)*	Tam Dao	July 2006	Leaves	94.5
9	MA02	*Mallotus apelta (Ma2)*	Tam Dao	December 2006	Leaves	92.5
10	MA03	Mallotus paniculatus	Tam Dao	April 2006	Leaves	58.4
11	SP4	*Mallotus sp2*	Langson	March 2006	Leaves	56.8
12	SP5	*Mallotus philippinensis*	Langson	March 2006	Leaves	98.9
13	MA11	*Mallotus macrostachyus*	Langson	March 2006	Leaves	75.7
14	MA12	*Mallotus microcarpus*	Quangbinh	March 2006	Leaves	83.1
15	MA13	*Mallotus pallidus*	Quangbinh	March 2006	Leaves	65.3
16	**MA14**	***Mallotus oblongifolius***	**Quangtri**	**March 2006**	**Leaves**	**6.7**
17	**MA15**	***Mallotus floribundus***	**Langson**	**November 2006**	**Leaves**	**6.4**
18	MA16	*Mallotus cuneatus*	Langson	November 2006	Leaves	86.9
19	**MA17**	***Mallotus cuneatus***	**Quangbinh**	**December 2006**	**Leaves**	**10.3**
20	MA18	*Mallotus sp3*	Quang tri	December 2006	Leaves	91.6
21	MA19	Mallotus yunnanensis	Lang Son	November 2006	Leaves	91.6
22	MA20	*Mallotus poilanei*	Ke Bang	March 2006	Leaves	90.5
23	MA22	*Mallotus hookerianus*	Dakrong	March 2006	Leaves	55.6
24	MA23	*Mallotus nanus*	Daclak	March 2006	Leaves	78.4
25	MA24	*Mallotus sp4*	Daclak	March 2006	Leaves	56.9
26	M25	*Mallotus oreophilus*	LaoCai	June 2006	Leaves	88.8
27	**MA28**	***Mallotus philippinensis***	**Cucphuong**	**December 2006**	**Leaves**	**22.3**
28	**MA29**	***Mallotus barbatus***	**Cucphuong**	**December 2006**	**Leaves**	**11.3**
29	MP31L	*Mallotus paniculatus*	VQG Pumat	September 2006	Leaves	73.5
30	MP32R	*Mallotus paniculatus*	VQG Pumat	September 2006	Roots	91.5
31	MP33L	*Mallotus paniculatus*	Bach Ma-TTH	October 2006	Leaves	81.5
32	MP34R	*Mallotus paniculatus*	Bach Ma-TTH	October 2006	Roots	83.5
33	**MP35R**	***Mallotus paniculatus***	**Cucphuong**	**December 2006**	**Roots**	**27.9**
34	MP36L	*Mallotus paniculatus*	Cucphuong	December 2006	Leaves	75.3
35	**MN37R**	***Mallotus nanus***	**VQG-Bachma**	**May 2006**	**Roots**	**5.0**
36	**MN37L**	***Mallotus nanus***	**VQG-Bachma**	**May 2006**	**Leaves**	**4.5**
37	MN39C	*Mallotus nanus*	VQG-Bachma	May 2006	Bark	76.8
38	M40L	*Mallotus sp5*	VQG Bavi	August 2006	Leaves	73.7
39	M41C	*Mallotus sp6*	VQG Bavi	August 2006	Bark	65.6

the plants metabolite profile in its chromatographic fingerprint. So far, high-performance liquid chromatography (HPLC) and gas chromatography (GC) (for volatile components) have been mainly used for this purpose [6].

Our research group developed a screening method for possible antioxidant compounds, using a multivariate calibration model that links chromatographic fingerprints to antioxidant activity [7]. From the various regression modeling techniques tested, using HPLC as the chromatographic fingerprinting technique, and a 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity test (antioxidant activity test on the plant extract) as a reference method to build the multivariate calibration models, PLS and o-PLS proved to be the most suitable. MS/MS analysis of the indicated peaks is currently under study and revealed that some of the indicated peaks did not correspond to one, but to two or more unseparated compounds. This suggests that the separation power of the HPLC–UV method is to some extend limited to be used as a screening method for antioxidant compounds in *Mallotus* samples. This encouraged us to assess whether pCEC, a miniature separation technique, can bring an added value. Its high resolution potential and minimal solvent consumptions might be beneficial for such screening applications that need to resolve many complex samples.

Just as in Ref. [7], diverse *Mallotus* species were taken as model plant extracts for the study. To obtain a data set containing metabolites as diverse as possible, various *Mallotus* species, from different plant parts, originating from numerous cultivation places and gathered during various harvest seasons were purposely chosen (Table 1). The plant genus *Mallotus* is not commonly known in Western countries, nevertheless, it has a long history of traditional use in Asian countries, such as China and Vietnam. Roots and leaves from *Mallotus apelta*, for instance, have been used for the treatment of chronic hepatitis [8]. A number of papers have been published on the pharmacological effects and composition of several *Mallotus* species [9–15].

Mainly driven by environmental issues and increasing costs of solvents and their disposal, miniaturization has become one of the major trends in chromatographic science. Also, because of practical limitations related to pressure and column length, the maximum efficiency that can be obtained in conventional HPLC is constrained to a certain extent. Recent developments in HPLC, such as long (or coupled) monolithic silica columns [16,17], high-temperature LC (HTLC) [18] and ultra-(high) performance LC (U(H)PLC) [19–21] have the potential to increase peak efficiency or peak capacity, each in their own way. For this reason, they can be very useful in applications that need to resolve very complex samples. For these techniques, there is also a trend towards miniaturization, as the impact of frictional heating can be minimized when working with small diameter columns. Alternatively, columns possessing diameters as small as capillaries have the advantage that practitioners are not anymore restricted to use pressure as the only driving force of the mobile phase (MP). Capillary electrochromatography (CEC) uses solely an electro-driven force to drag the MP through such a capillary column. The presence of an electro-osmotic flow (EOF) is thought to be responsible for flattening the flow profile compared with solely pressure driven flow [22]. Although this can yield efficiencies as high as in capillary electrophoresis (CE), there

is a practical limit to the applied voltage because of bubble formation and problems related to Joule heating. Instead, pressurized CEC (pCEC) aims to combine voltage and pressure in a way that negative effects related to both voltage and pressure can be controlled while high efficiencies still can be obtained [23,24]. Indeed, the technique sacrifices some efficiency compared to CEC, however gains some speed and reproducibility due to the added pressure. It was also found that the electric field affects the partitioning of the solutes and their retention [25–27].

A number of reports have demonstrated that highly efficient separations can be obtained with (p)CEC [28,29], making the technique potentially useful for applications that need to resolve very complex samples, *i.e.* mixtures of closely related compounds. So far, most papers on plant analysis with (p)CEC have been focusing on the quantitative analysis of known components of the plant [30–38]. Though interesting, only a few papers on metabolic fingerprinting of plant extracts with (p)CEC have been published [39,40]. Fingerprints of extracts of flos *Carthami* [40] and rhizoma *Chuanxiong* [39] were developed with commercially available pCEC instruments and commercial particle-packed C18 capillary columns. In both studies, the pCEC fingerprints revealed more characteristic peaks than those developed with a conventional HPLC method using a C18 (250 mm × 4.6 mm i.d., 5 μm) column. pCEC fingerprints were found to distinguish substitutes and adulterants of flos *Carthami* and they allowed discriminating samples of rhizoma *Chuanxiong* from different sources. However, from the tested criteria, HPLC remained superior in precision and what the authors called "stability of the analyses".

In the present paper, it is assessed whether pCEC can bring an added value in a screening for antioxidant compounds in extracts from *Mallotus* species, *i.e.* in terms of separation power necessary to indicate possible antioxidant compounds and of solvent consumption. Hereby, the previously developed HPLC method from Ref. [7] is considered as a benchmark. It is also being critically assessed whether the technique can meet the fingerprinting requirements for the present application, *i.e.* in terms of sensitivity, precision and column robustness. To get a complete view of the techniques potential for this screening application, encountered benefits and concerns are reported and discussed.

2. Methods and materials

2.1. Chemicals and reagents

HPLC grade methanol, acetonitrile (ACN) (both Fisher Scientific, Leicestershire, UK), trifluoroacetic acid (TFA) (Sigma–Aldrich, Steinheim, Germany) and water, obtained from a MilliQ purification system (Millipore, Bedford, MA, USA), were used to prepare the mobile phases. Phenol (Merck, Darmstadt, Germany), toluene (Sigma–Aldrich) and caffeine (Fluka, Buchs, Switzerland) were used in a mixture to test the column performance. Thiourea (Merck) was used as dead time marker of the pCEC experiment.

2.2. Herbs and preparation of the herbal extracts

39 *Mallotus* crude samples, from at least 17 different species, were collected in different Vietnamese regions (Table 1). Six samples were unidentified. Depending on the sample, the leaves, roots or bark were used. All samples were authenticated by Professor Nguyen Nghia Thin (Hanoi National University, Vietnam) and voucher specimen deposited at the Institute of Natural Products Chemistry, Hanoi, Vietnam. Extracts were prepared by weighing 2.5 g plant sample and extracting three times with 25 mL methanol in an ultrasonic bath (Branson Ultrasonic Corporation, Connecticut, USA) at a temperature between 40 and 50 °C during 1 h. The extract was filtered through a 0.24-μm pore size filter paper (Whatman, Hanoi, Vietnam) and evaporated at reduced pressure (60 Pa) and elevated temperature (50 °C). The obtained extracts were divided over three sample tubes, *i.e.* one for the chromatographic analyses, one for the DPPH radical scavenging assay and one as a library sample.

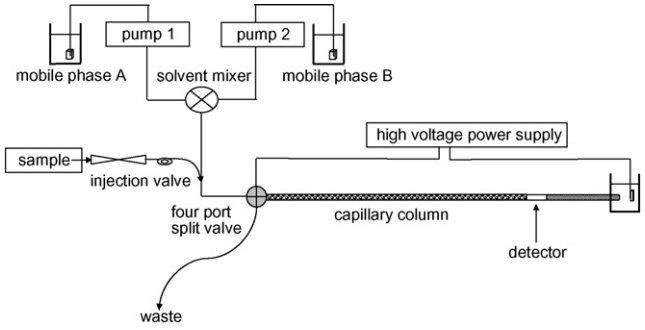

Fig. 1. Set-up of the pCEC instrument.

2.3. pCEC experiments

2.3.1. Apparatus

pCEC experiments were performed on the presently commercially available pCEC instrument [41], a Trisep™-2100 capillary electrochromatography system (Unimicro Technologies, Pleasanton, CA, USA). A schematic representation of the apparatus is shown in Fig. 1. The apparatus comprised a Unimicro binary HPLC pump, a high voltage power supply, a Valco six-port injection valve, a UV–Vis variable wavelength detector equipped with a cell for on-column detection, and a Unimicro Trisep™ workstation 2003. A gradient mixer merged the two solvent flows coming from pumps 1 and 2 in order to deliver a continuous gradient mobile phase flow. Samples were injected manually through the injection valve, subsequently introduced in the internal nano-liters sample loop with fixed volume, and then carried by the mobile phase flow to the four-port split valve. After splitting in this valve, a fraction of the initial flow enters the capillary column under a constant pressure range of 70–75 bar. During separation, pressure and a high voltage of 8 kV were simultaneously applied to the column. The high voltage was applied to the outlet reservoir, and the body of the split valve was grounded. Commercially available packed capillary columns (100 μm i.d. × 375 μm o.d., 45 cm total length), containing a 20 cm section packed with 3 μm C18 silica particles, were purchased by Unimicro Technologies, CA, USA [41].

2.3.2. Conditions

The 39 *Mallotus* samples were analysed on the same column using a solvent gradient program. The mobile phase contained (A) water + 0.05% TFA and (B) ACN + 0.05% TFA. The solvent gradient program comprised three steps, *i.e.* 0–25 min: 5–20% B, 25–50 min: 20–95% B, 50–60 min: 95% B. Mobile phases were filtered through a 0.2-μm Teflon® membrane filter (Pall Corporation, Ann Arbor, MI, USA) and degassed during 15 min on an ultrasonic bath (Branson Ultrasonic Corporation, Danbury, CT, USA) prior to analysis.

Between runs the system and column were rinsed with 2-propanol for at least 1 h, as well as overnight. After each analysis, the injector loop was rinsed manually with methanol. Every six analyses, a column performance test was performed, using an isocratic mobile phase containing water/ACN, 50/50 (v/v). The total flow rate of the two pumps was always set on 0.100 mL/min and detection was performed at a wavelength of 254 nm.

2.3.3. Sample preparation

Samples for HPLC analysis were prepared diluting 50.0 mg crude extract in 2.0 mL methanol. The solution was mixed during 15 min at 400 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 through a 25-mm syringe polypropylene membrane with 0.2-μm pore size (VWR International, Leuven, Belgium). 1.5 mL of the prepared sample solution was taken and dried under a stream of nitrogen. Immediately before injection, the residue was redissolved in 300 μL of methanol and filtered two times through a 25-mm syringe polypropylene membrane with 0.2-μm pore size (VWR International, Leuven, Belgium).

2.4. HPLC experiments

The HPLC experiments used for comparison purposes are described in Ref. [7].

2.5. DPPH radical scavenging test

The DPPH antioxidant activity scavenging test measures the capacity of the *Mallotus* crude extract to scavenge the stable 1,1-diphenyl-2-picrylhydrazil radical (DPPH•). In its radical form, DPPH has an absorption band at 515 nm, which disappears upon reduction by an antiradical compound. The remaining DPPH• concentration in the reaction medium is then estimated from a calibration curve. The percentage of remaining DPPH• (%[DPPH•$_{rem}$]) is expressed as follows:

$$\%[\mathrm{DPPH^{\bullet}}_{rem}] = \frac{[\mathrm{DPPH^{\bullet}}_{20\,min}]}{[\mathrm{DPPH^{\bullet}}_{0\,min}]} \times 100 \quad (1)$$

where [DPPH•$_{0\,min}$] is the starting concentration of DPPH radicals, and [DPPH•$_{20\,min}$] the remaining concentration after 20 min of incubation with the sample at a concentration of 20 μg/mL.

The DPPH radical scavenging test was performed as described in Ref. [42]. The reported results (Table 1) are the averages of three independent measurements.

2.6. Data analysis

Computations were performed using Matlab™ 7.1 (The Mathworks, Natick, MA). All data (pre-)processing was performed using in-house written m-files.

2.6.1. The linear multivariate calibration model to indicate antioxidant compounds

A linear multivariate calibration model is built to indicate those peaks in the fingerprints potentially responsible for the antioxidant activity of a measured sample. The model links chromatographic fingerprints (data matrix **X**) of 39 *Mallotus* samples to a response vector **y**, representing the DPPH radical scavenging activity test results (Eq. (2)).

$$\mathbf{y} = \mathbf{Xb} + \mathbf{f} \quad (2)$$

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

No division of the data set was made into a calibration and a test set because the data set was considered not large enough and secondly prediction of the antioxidant activity of new samples was not the aim of the study. The data set also contains too few samples with antioxidant activity versus too many samples without to be appropriate as a calibration set for quality prediction purposes.

By means of examining the regression coefficients (**b**) of the model, peaks corresponding to potential antioxidant compounds (or to those representing a similar behaviour *i.e.* that are present at high concentration when the antioxidant activity is high) can be indicated.

2.6.2. PLS

Different multivariate calibration techniques can be used to express the relationship between **X** and **y** [43,44]. It was previously observed on HPLC fingerprints of the same *Mallotus* samples that Multiple Linear Regression (MLR) and Principal Components Regression (PCR) made an inadequate variable selection and/or took large variations uncorrelated to the antioxidant activity into account [7]. The best models were obtained using partial least squares (PLS) regression. PLS uses a non-linear iterative partial least squares (NIPALS) algorithm to create orthogonal latent variables that maximize the co-variance between **y** and **X** [43]. Especially Orthogonal Projections to Latent Structures (o-PLS) regression models were found to give the simplest models with high interpretability [7–46]. o-PLS makes use of a modified NIPALS algorithm, also removing information in **X** not correlated to **y**, and this way reducing the model complexity [45]. Therefore, in this paper, PLS and o-PLS are the applied regression techniques.

2.6.3. Data pre-processing

Prior to multivariate data analysis, data pre-processing is commonly used to make the extraction of latent variables easier [43]. In general, when working with chromatographic data, it is recommendable to align the fingerprints since peak shifts may occur due to column ageing and small variations in mobile phase composition, flow rate or temperature [43]. However, because of the very diverse data set in this application, *i.e.* different *Mallotus* species, alignment of the fingerprints was not considered appropriate [7]. Mean-centering was applied to correct for any off-set in the electro-chromatograms, while variability between electro-chromatograms, *e.g.* due to small variations between injections, was reduced by normalisation.

3. Results and discussion

3.1. Transfer of the HPLC method to pCEC

A generic 60-min run time HPLC method [7] on two serial coupled Chromolith™ performance RP-18e columns (100 mm × 4.6 mm i.d.) and a Chromolith™ RP-18e guard column (5 mm × 4.6 mm i.d.), was transferred to pCEC. As it was the purpose to transfer the method to pCEC with conditions as close as possible to the original HPLC method, it was chosen to retain the original mobile phase composition, gradient conditions, column length and stationary phase C18 chemistry. The flow rate was adjusted to the miniature column geometry, *i.e.* it was set on 0.1 mL/min instead of 1 mL/min in HPLC, and a voltage of 8 kV was added as an additional driving force of the mobile phase. This way, the solvent consumption was decreased by a factor 10. No air bubbles or problems related to Joule heating were found when these conditions were applied.

3.2. Requirements of the analytical method

The present application needs ideally an analytical separation technique that generates highly efficient separations so that the model indicates peaks in the fingerprints only corresponding to a single compound. Furthermore, an acceptable sensitivity and precision of the analytical method deems important for the modelling of the fingerprints, and for screening purposes, a reasonable sample

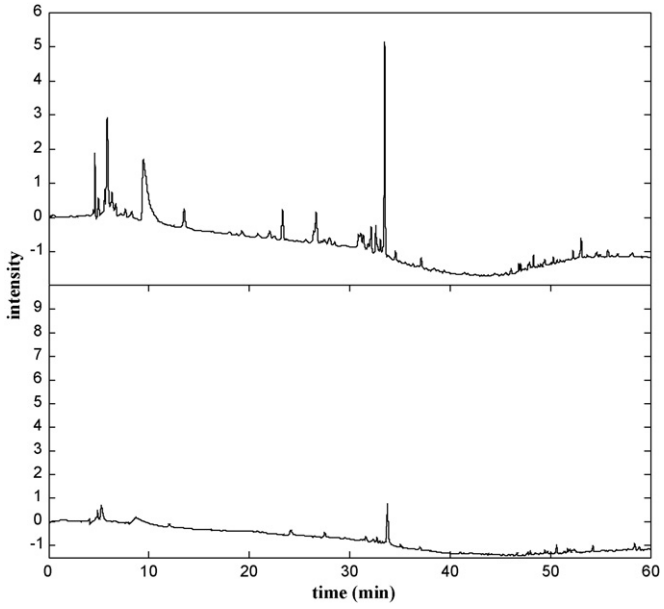

Fig. 2. (Down) pCEC fingerprint of *Mallotus paniculatus* – initial sample preparation (as for HPLC); (top) pCEC fingerprint of the same *Mallotus paniculatus* sample 5 times concentrated. Conditions: 3 μm C18 capillary (20 cm × 100 μm); mobile phase: gradient elution (A) water + 0.05% TFA, (B) ACN + 0.05% TFA: 0–25 min: 5–20% B, 25–50 min: 20–95% B, 50–60 min: 95% B; 0.100 mL/min; 8 kV; λ = 254 nm.

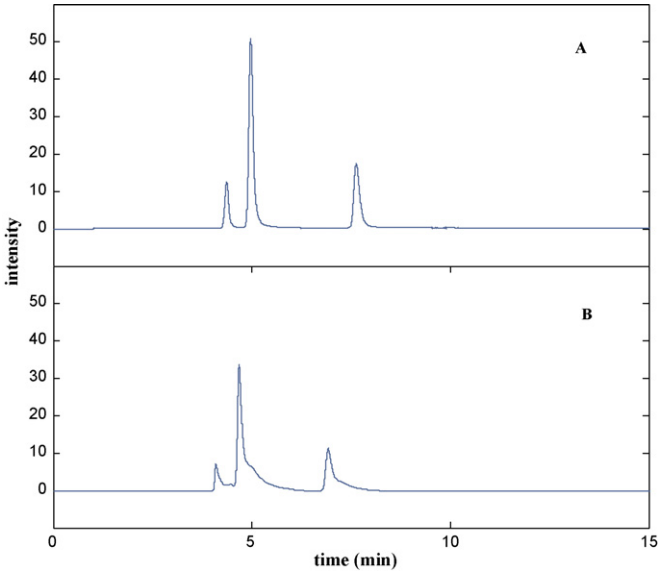

Fig. 3. Column performance test: 3 μm C18 capillary (20 cm × 100 μm), isocratic conditions, mobile phase water/ACN (50/50), flow 0.100 mL/min, UV 254 nm, mixture of 3 chemicals: 0.35 g phenol, 2 mL toluene, 135 mg caffeine. (A) Test performed on a new capillary; (B) test performed after 19 *Mallotus* samples analysed.

throughput and low solvent consumption is mostly desired. These requirements were assessed for pCEC in the present application.

3.2.1. Detection sensitivity of the pCEC method

Because of the well-known problem of low detection sensitivity when using capillary electro-separation techniques with on-line UV detection, the sample needed to be more concentrated than in the HPLC method. As can be derived from Lambert–Beer's law (Eq. (3)), the short optical path length that the capillary creates and the very low amounts of sample that can be loaded on the capillary both contribute to this problem.

$$A = \varepsilon C l \quad (3)$$

where A is the measured absorbance, ε (L/mol cm) the molar extinction coefficient, C the concentration of the analyte (mol/L), and l the optical path length (cm).

Concentrating the sample 5-fold increased the signal-to-noise ratios of the peaks in the pCEC fingerprints approximately 3–4 times, giving rise to fingerprints with acceptable detection sensitivity as is shown in Fig. 2.

3.2.2. Column robustness of the pCEC method

Initially, a procedure comprising 20 min rinsing with pure ACN followed by 20 min conditioning with a mixture of mobile phase A (MP A)/ACN 95/5 (v/v) was applied between the analyses. This rinsing, to maintain column robustness and to prevent carry-over, must ensure that no components of the extract remain on the column. This procedure was useful in preventing carry-over, since tests with a blank injection after analyses of diverse *Mallotus* samples displayed no peaks. However, the procedure proved not satisfying to maintain column robustness, since a strong decrease in column performance was noticed after only 19 runs. Moreover, a further examination of the capillary column revealed that the inlet frit was completely damaged.

The effect of the concentrated *Mallotus* samples on the stationary phase was evaluated by means of a column performance test. This test comprised the assessment of the separation of a mixture of chemicals, *i.e.* phenol, toluene and caffeine, at isocratic conditions with a water/ACN 50/50 (v/v) mobile phase in capillary liquid chromatography (CLC) mode (without voltage). From Fig. 3A, depicting the performance of a new capillary, and Fig. 3B, showing the performance of the same capillary column after 19 concentrated *Mallotus* samples were analysed, it is clear that efficiency and resolution were compromised and all peaks were tailing. To quantify the results, some system suitability (SS) parameters were assessed, being resolution (R_s), number of theoretical plates (N), and tailing factor (Table 2). After merely 19 sample runs, no parameters met the a priori defined SS criteria anymore. This dramatic decrease in column performance is most probably due to the analysis of highly concentrated *Mallotus* extracts on columns

Table 2
Column performance test for a new capillary column and for the same column after 19 *Mallotus* sample runs: separation of phenol, toluene and caffeine evaluated by system suitability criteria. Conditions: 3 μm C18 capillary (20 cm × 100 μm), mobile phase MilliQ/ACN 50/50, flow 0.100 mL/min, UV 254 nm.

System suitability parameters	System suitability criteria[a]	Performance of new column			Performance of column after 19 Mallotus sample runs		
		1[b]	2[b]	3[b]	1[b]	2[b]	3[b]
Retention time (min)		4.1	4.6	6.7	4.1	4.7	6.9
R_s	>2	–	6.53[c]	9.66[d]	–	1.68[c]	8.59[d]
N	>2000	9167	9838	14,371	2869	952	3611
Tailing factor	≤2	0.93	1.24	1.22	2.36	3.78	3.66

[a] System suitability recommendations [47].
[b] Peak number
[c] Critical peak pair 1/2.
[d] Critical peak pair 2/3.

Table 3
The monitored column performance of the improved method on the same capillary column: separation of phenol, toluene and caffeine evaluated by system suitability criteria. Conditions: 3 μm C18 capillary (20 cm × 100 μm), mobile phase water/ACN 50/50 (v/v), flow 0.100 mL/min, UV 254 nm.

	$R_s > 2$		$N > 2000$			Tailing factor ≤ 2		
	1–2[a]	2–3[a]	1[a]	2[a]	3[a]	1[a]	2[a]	3[a]
New capillary	2.40	9.59	9013	7555	11,368	1.31	1.49	1.57
After 6 sample runs	2.67	10.82	8997	7415	11,621	1.38	1.52	1.55
After 12 sample runs	3.63	12.01	9381	11,851	12,220	1.29	1.41	1.50
After 18 sample runs	3.59	13.64	13,846	13,882	18,351	1.01	1.11	1.10
After 24 sample runs	3.30	11.06	12,384	13,136	17,517	1.02	1.11	1.12
After 30 sample runs	3.32	13.22	12,934	12,984	17,626	1.12	1.14	1.16
After 36 sample runs	3.83	13.58	13,489	13,298	17,594	1.02	1.12	1.14
After 42 sample runs	3.78	14.71	12,457	12,541	15,722	1.00	1.15	1.16
After 54 sample runs	3.46	13.44	12,582	12,839	16,746	1.15	1.16	1.19

[a] Peak number.

having miniaturized geometries, and the current unavailability to use guard columns in pCEC.

Since it was preferred for the chemometric modeling that the calibration model is made from a full data set analysed on the same column, attempts were made to make the column lifetime longer. Analysing a data set on different columns creates an extra source of variability, which one tries to avoid. Since the currently available equipment lacks guard columns and uses capillary columns having modest robustness, we chose to apply extensive rinsing procedures and longer column regeneration times to increase the column lifetime. Even if this is at the expense of time and solvent consumption, it is currently the only option to test the potential of pCEC for this application.

In practice, the herbal samples were filtered twice before injection, and extensive rinsing procedures of 60 min with 2-propanol were applied between sample runs. A strict performance testing of the column, *i.e.* every six samples, allowed controlling the behaviour of the chromatographic system. This way, corrective actions, such as backward rinsing of the column, to regenerate it, could be taken when needed. The latter approach deemed necessary to fingerprint the whole *Mallotus* sample set of 39 samples on the same capillary column. Table 3 shows the performance, expressed as SS parameters, of the optimized pCEC method. Now, even after 54 runs of concentrated samples, the stationary phase still showed an acceptable performance, meeting all the SS criteria. Consequently, the same capillary and method could be used to generate the fingerprints from the *Mallotus* data set.

In conclusion, some problems were encountered when analysing highly concentrated *Mallotus* extracts without the availability of guard columns. These were related to the known low detection sensitivity in UV and the modest robustness of the capillary columns. Although these issues could be circumvented by optimized rinsing procedures, they limit the benefit of the reduced solvent consumption and the sample throughput to some extent. Hypothetically, this issue may be overcome by using other detection techniques than UV.

3.2.3. The resolution and fingerprinting power of the pCEC method

To get an idea of the fingerprinting performance of pCEC, pCEC fingerprints were compared to those generated with the HPLC method [7]. For this screening application, a higher separation power might be favourable for the indication of possible antioxidant compounds in the extract. Chromatographic separation can be expressed by different parameters, *e.g.* selectivity, retention, efficiency and peak capacity. Selectivity and retention are strongly method dependent, and can be optimized easily by altering the method's conditions. Since the methods used here are generic methods to fingerprint different *Mallotus* samples, and it was attempted to evaluate the separation power of the techniques and not the method performances, method parameters influencing the separation power were taken as much as possible similar in HPLC and pCEC conditions. Thus, gradient conditions, mobile phase compositions, chemistry on the stationary phase (C18) and column length were chosen the same in both pCEC and HPLC methods. The temperature was an uncontrolled parameter in pCEC, while in the HPLC method separations were performed at a constant temperature of 25 °C.

Similar to HPLC, pCEC was able to display characteristic chromatographic fingerprints of the various *Mallotus* samples (Fig. 4). Retention times of the major peaks in pCEC varied from those in HPLC. However, there is no trend since the retention times are sometimes either earlier or later than the corresponding peaks in the HPLC chromatogram. This is presumably due to the different chromatographic configurations inherent to both techniques and the added voltage in pCEC, as is theoretically expected.

To evaluate the separation power of pCEC fingerprints developed in gradient conditions, peak capacity is considered the most suited parameter. Peak capacity is a theoretical term, corresponding to the number of peaks in a chromatogram that can be separated. It not only depends on the technique itself, but also on parameters such as gradient time, mobile phase flow rate and column length. Generally, the highest peak capacities can be yielded on long columns but they require long analysis times. The same column length for separation (20 cm) was used in both techniques to make the comparison of the techniques easier. It should be noted that no attempts were made to reduce the analysis time, since this was not the goal of this study. It was rather the intention to evaluate

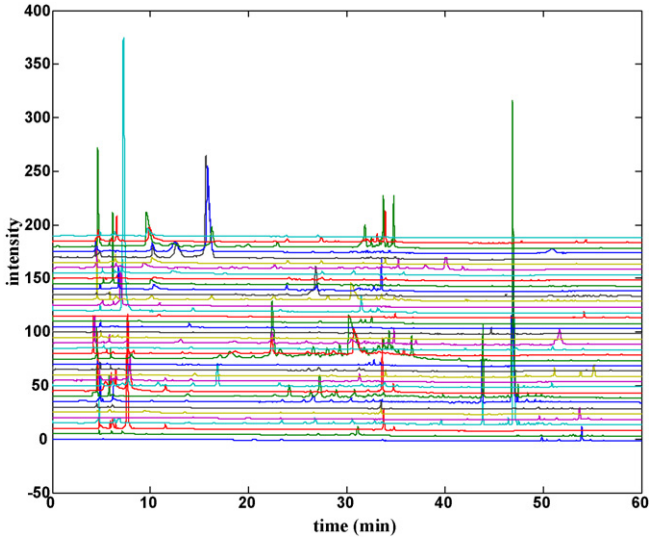

Fig. 4. Fingerprints of the 39 *Mallotus* extracts obtained with pCEC.

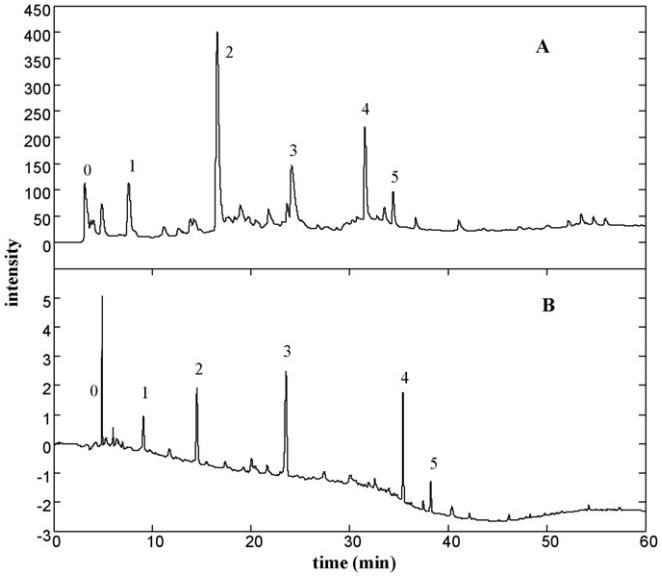

Fig. 5. Fingerprint chromatograms of *Mallotus cuneatus* (sample 19) obtained with (A) the HPLC method; column: 2 coupled Chromolith Performance RP-18e (100 mm × 4.6 mm) + Chromolith RP-18e guard column (5 mm × 4.6 mm), mobile phase: gradient elution water + 0.05% TFA/ACN + 0.05% TFA, T = 25 °C, λ = 254 nm, flow rate: 1.0 mL/min. (B) The pCEC method: column: RP-3 μm C18 capillary (20 cm × 100 μm), flow: 0.100 mL/min, mobile phase: gradient elution water + 0.05% TFA/ACN + 0.05% TFA, 8 kV, λ = 254 nm, injected sample 5× more concentrated than in HPLC.

the separation potential of pCEC on a standard 60-min gradient run. Peak capacities (P) were estimated from the average peak width of an (electro) chromatogram and the gradient run time (t_g) [48]:

$$P = 1 + \frac{t_g}{(1/n)\sum_1^n w} \quad (4)$$

where n is the number of peaks selected for the calculation and w are the widths of the selected peaks. Being a representative example, Fig. 5 compares fingerprints from *Mallotus cuneatus* (sample 19 of the data set) developed with HPLC (A) and pCEC (B). To calculate P, the indicated peaks 1–5 in Fig. 5 were considered. Since they are distributed uniformly throughout the chromatogram a representative average estimation of the peaks widths can be obtained. Widths of these peaks were determined via the magnitude-concavity method [49], being less sensitive to baseline errors, peak overlap and peak asymmetry than other methods.

In the HPLC chromatogram (Fig. 5A), a peak capacity of 344 was calculated considering the five indicated major peaks, while a peak capacity of 806 was found in the pCEC electrochromatogram (Fig. 5B). The very efficient separations in pCEC are thought to be the result of the EOF contribution to the driving force flattening the flow profile and the on-line UV detection. Despite our expectations, the higher peak capacity of pCEC did not result in a higher number of major peaks in the pCEC fingerprints when compared to the HPLC fingerprints. Similar results were observed for the majority of the other tested *Mallotus* samples. Thus, in case of the tested *Mallotus* samples, pCEC is more likely to resemble the fingerprinting performance of the compared HPLC method than to surpass it.

3.2.4. Precision of the pCEC method

Precision of the pCEC method, involving sample preparation and chromatographic fingerprinting, was determined using an experimental set-up where each day two *M. cuneatus* (sample 19) samples were analysed during six subsequent days. ANOVA was then used to determine the repeatability and time-dependent intermediate precision on the retention times of the major peaks in the fingerprint chromatogram. Since it is not our intention to use the method quantitatively, precision on the peak areas was not considered. In this application, it must be emphasized that a low variability in retention times of the peaks may be very important since alignment of the peaks prior to data analysis is not so evident. The reasons are the very diverse set of *Mallotus* samples generating different fingerprints and the fact that no identification of the peaks is possible in this (stage of the) study, using a single wavelength UV detector. Thus, it becomes complicated to decide which peaks correspond to the same compounds in the fingerprints and should be aligned. The risk of aligning wrongly is here very high, and therefore not considered useful.

Table 4
Precision (% RSD) of the retention times in the pCEC method for five different peaks in the fingerprint chromatogram of *Mallotus cuneatus*.

	Repeatability (%)	Intermediate precision (%)
Dead time peak (peak 0)	0.2	8.3
Peak 1	4.3	15.6
Peak 2	1.6	24.0
Peak 3	0.4	8.2
Peak 4	0.2	21.8
Peak 5	0.6	25.5

To estimate the precision the whole sample preparation procedure and subsequent pCEC analysis were considered. The precision was investigated on the retention times of the dead time peak (peak 0), representing compounds having no affinity for the stationary phase, and of the five peaks indicated over the fingerprint chromatogram (see Fig. 5) of *M. cuneatus* (sample 19). Table 4 shows the precision results of the pCEC method on this *Mallotus* sample. The repeatability of the optimized pCEC method was less than 5% for all tested peaks, indicating an acceptable repeatability. The time-dependent intermediate precision varied from 8.2% up to values as high as 25.5%. Surprisingly, this did not cause problems when modelling the pCEC fingerprints (see Section 3.3).

3.3. Modeling the pCEC fingerpints

3.3.1. Antioxidant activity of the Mallotus samples

The antioxidant activity was measured by means of a DPPH radical scavenging test. The results are shown in Table 1. Eight samples of the *Mallotus* sample set were considered as being highly antioxidant (%$DPPH_{rem}$ < 30), *i.e.* samples 16, 17, 19, 27, 28, 33, 35 and 36. The other samples showed less antioxidant activity according to the results of the DPPH test. Their %$DPPH_{rem}$ results vary between 51.1 and 100.0. The arbitrary threshold of %$DPPH_{rem}$ > 50 was set to designate samples as being 'non-active'. Hence, 31 samples could be classified as being 'non-active' against eight samples being 'highly active'. Interestingly, samples from the same *Mallotus* species but obtained in different conditions can show major differences in antioxidant activity. *Mallotus philippinensis* leaves, for example, possessed high antioxidant activity when collected in Cucphuong and collected in December 2006 (%$DPPH_{rem}$ 22.3), however showed no antioxidant activity (%$DPPH_{rem}$ 98.9) when harvested in Langson in March 2006.

3.3.2. PLS models

Since the main aim of this work is to test potential of pCEC in a specific application, *i.e.* the screening for antioxidant compounds in plant extracts from *Mallotus* species, the antioxidant activity of the 39 *Mallotus* samples was modelled as a function of their pCEC fingerprints. Leave-one-out cross-validation (LOOCV) was applied to select the optimal model complexity [R44]. Fig. 6A shows the root mean square error of cross validation (RMSECV), describing the pre-

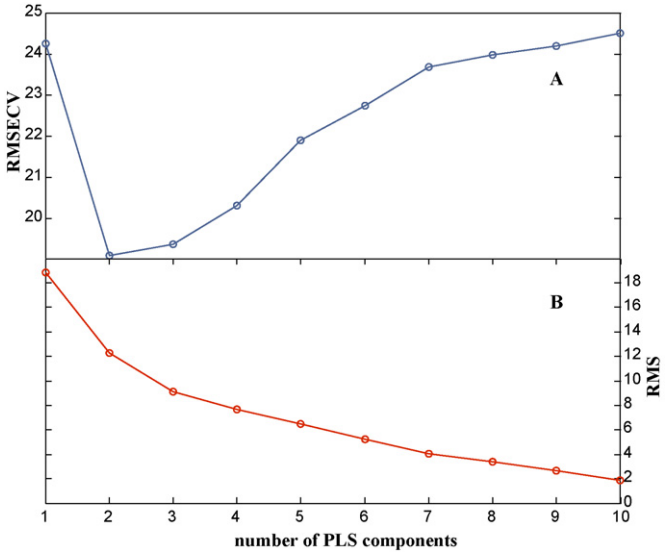

Fig. 6. (A) RMSECV versus the number of PLS components; (B) RMS versus the number of PLS components (for the 10 first PLS components).

dictive ability of the models, and Fig. 6B the residual mean squares (RMS), describing the model fit, of the PLS models with different numbers of PLS components. A PLS model with two PLS components has the best predictive ability (lowest RMSECV) but has a relative low model fit (high RMS). This model would be preferred when used for quality control purposes of new *Mallotus* extracts, where one would use fingerprints of the plant extracts to predict their antioxidant activity.

In this application, however, we will use the model not for the prediction of future samples, but to link the regression coefficients to given peaks in the fingerprint chromatograms. The latter might be easier from a better fitting model. When considering three PLS components in the model, the model fits better due to a reduced RMS value, while the RMSECV value is only slightly increased. Because of this good compromise between model fit and predictive ability, the three-component PLS model was preferred in this application and will be further referred to in this paper as the PLS model.

Since o-PLS gave rise to a better interpretability of the regression coefficients in Ref. [7], this technique was also applied here. A lower model complexity was obtained by the removal of orthogonal variation out of the PLS model, however, the o-PLS model showed a poorer prediction ability (RMSECV 27.0) in comparison to the above PLS model.

Samples were labelled as 'highly antioxidant' or as 'non-active' when the %DPPH values were <30 and >50, respectively, resulting in eight 'highly antioxidant' (16, 17, 19, 27, 28, 33, 35 and 36) and thirty-one (the others) 'non-active' samples. Table 5 shows for the eight highly antioxidant samples the predicted activity by both the PLS and o-PLS model. The PLS model shows a good predictive ability since it predicts seven out of eight as highly antioxidant. Only for sample 33 a value of 37.7 was obtained, which means that this model predicts this sample as a borderline case or as intermediate antioxidant (30 < %DPPH < 50). No antioxidant activity was predicted for the non-active samples by the model. The low bias to predict antioxidant samples (5.3) suggests an acceptable reliability of the regression coefficients.

The o-PLS model, for comparison, labelled three of the eight highly antioxidant samples wrongly. Samples 19 and 33 were predicted as intermediate antioxidant, while sample 28 was predicted as non-active. Furthermore, from the non-active samples the o-PLS model labelled samples 25 and 37, being non-active, as highly

Table 5
Results from the DPPH radical scavenging assay and predictions from the PLS and o-PLS models built from the pCEC fingerprints. Preprocessing: normalisation and mean centering.

Sample no.	DPPH	PLS	o-PLS
16	6.7	−1.9	−21.6
17	6.4	8.9	18.7
19	10.3	15.5	34.2
27	22.3	20.3	10.4
28	11.3	15.4	50
33	27.9	37.7	43.2
35	5	0.7	13.6
36	4.5	−1.7	9.5
Mean bias[a]	–	5.3	18.0
25	56.9	60.0	29.0
37	76.8	76.9	20.0

[a] For highly antioxidant samples.

antioxidant (Table 5). The prediction of the highly antioxidant samples also shows a larger bias (18.0) compared to the PLS model (5.3).

3.3.3. PLS score plots

The PLS score plot from the first two PLS components (Fig. 7), describing together 47% of total variance, discriminates the samples with high antioxidant activity. Indeed, pCEC fingerprints from samples with high antioxidant activity led to negative scores on both PLS components 1 and 2.

3.3.4. Regression coefficients to indicate possible antioxidant compounds

The regression coefficients (**b**) of the model can be used to indicate peaks corresponding to antioxidant compounds or to compounds presenting a similar concentration behaviour, *i.e.* which are present at high concentration when the antioxidant activity is high, and vice versa. Chromatographic peaks, for which the regression coefficient peaks of the model are largely negative, contain such compounds. This is because the DPPH radical scavenging test involves decreased DPPH values with increasing antioxidant activity. When regression coefficient peaks are largely positive, the

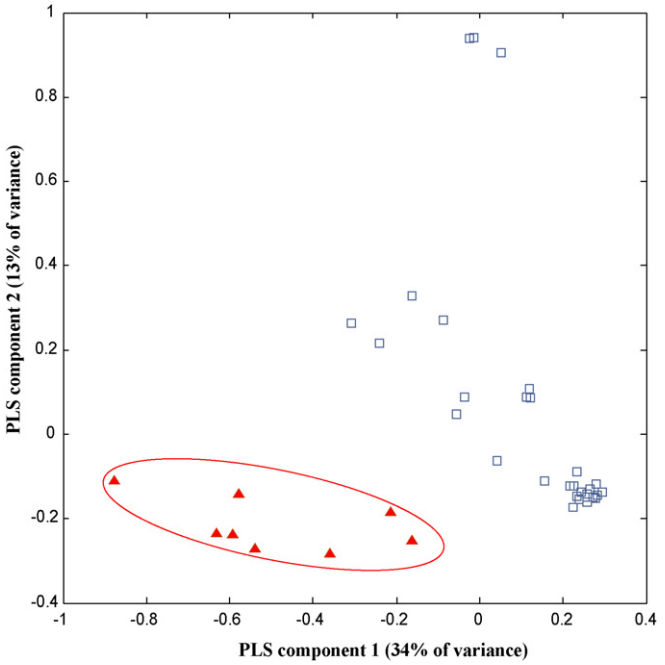

Fig. 7. Score plot of the first two PLS components. ▲, *Mallotus* samples with strong antioxidant activity; □, *Mallotus* samples with no or weak antioxidant activity.

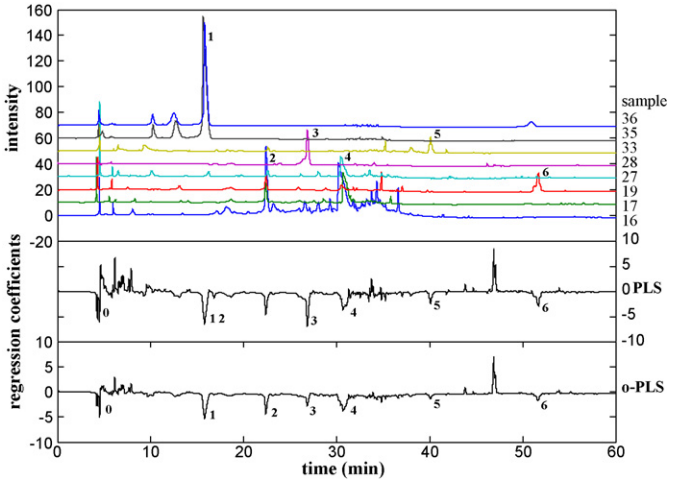

Fig. 8. Plot of the pCEC fingerprints of the antioxidant *Mallotus* samples. The two bottom plots show the regression coefficients from the PLS and o-PLS models, pre-processed with normalisation and column centering.

corresponding fingerprint peaks are supposed to match up to those compounds in the sample having an opposite behaviour to the antioxidant activity.

The regression coefficient plots (Fig. 8) of both the PLS and o-PLS model seem to confirm each other. Obviously, removing orthogonal information from the PLS model did not result in improved interpretability. As the PLS model has also a better predictive ability this model can be considered more suitable for this application.

The same six major negative peaks can be distinguished in both regression plots (Fig. 8). It is supposed that the peaks in the *Mallotus* fingerprints corresponding to them represent compounds having a significant contribution to antioxidant activity. Indeed, these six peaks correspond to retention times of peaks observed in the electro-chromatograms of one or more of the eight antioxidant *Mallotus* samples (samples no. 16, 17, 19, 27, 28, 33, 35 and 36), as can be seen in Fig. 8. Whether these compounds are truly active should, of course, be confirmed by testing the activity of the indicated fractions, but this was out of the scope of this study.

The peak 0 of the regression coefficients of the models is also negative. It was confirmed using the dead time marker thiourea that this peak corresponds to the peak representing the dead time in the pCEC fingerprints. The models indicated this peak because it is present when antioxidant activity is high. Thus, it is apparent that hydrophilic components, having no affinity for the C18 stationary phase, were also present in the highly antioxidant samples. Whether some of them have an antioxidant activity, should again be confirmed by an activity test.

The coefficient peak 4 is splitted, which may suggest that this peak corresponds to different antioxidant compounds in the fingerprints, or that this is caused by experimental shifts in retention time of one compound. The identity of the compounds corresponding to the indicated peaks, and whether they are truly active, should be confirmed by MS and/or NMR analysis of the indicated peaks.

The most preferred model in [7], *i.e.* the o-PLS model constructed from HPLC fingerprints, indicated a similar number of peaks corresponding to possible antioxidant compounds in the *Mallotus* extracts (Fig. 9). Thus, from these results it is apparent that the PLS and o-PLS models from the pCEC fingerprints have similar potential than the o-PLS model from HPLC fingerprints to indicate potential antioxidant compounds in *Mallotus* fingerprints.

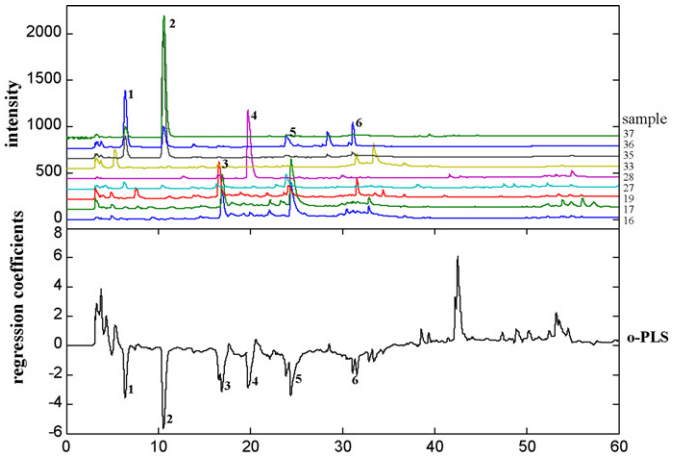

Fig. 9. Plot of the HPLC fingerprints of the antioxidant *Mallotus* samples. The bottom plot shows the regression coefficients from the o-PLS model [R24]. Pre-processing: normalisation and column centering.

4. Conclusions

In this paper, the potential of pCEC for the presented screening application was assessed. Taken into account its known features, *i.e.* in terms of improved separation power to indicate possible highly antioxidant compounds and reduced solvent consumption, it was evaluated whether pCEC can have a possible added value over HPLC. Considering its drawbacks, sensitivity, precision and column robustness were critically assessed to evaluate its overall potential for the presented application.

As theoretically expected, pCEC fingerprints of *Mallotus* extract samples showed a much higher peak capacity than those obtained with HPLC. But in spite of this benefit, a similar number of characteristic peaks were seen in most pCEC and HPLC fingerprints of the same *Mallotus* extracts. As a result, the regression coefficients of the PLS models from both HPLC and pCEC fingerprints indicated a comparable number of major regression peaks, possibly corresponding to antioxidant compounds.

Known drawbacks of pCEC, *i.e.* low detection sensitivity in UV and modest column robustness and precision could be circumvented by concentrating the samples and using optimized rinsing procedures. A reduced solvent consumption remains a benefit of pCEC over HPLC. However, the need for more extensive rinsing procedures limits this gain to some extent, even as the sample-throughput. Although intermediate precision of the pCEC fingerprints was high, this proved not to hinder the modeling.

For the current screening application, pCEC did not show the expected benefit in terms of high resolution power and performance to indicate highly antioxidant molecules in the *Mallotus* fingerprints. Because of its potential similar to HPLC, pCEC may still suit as a viable alternative technique for the considered screening application, but will be less preferred because of the need for more system maintenance and lower sample throughput.

Acknowledgements

Sigrid Pieters is a PhD fellow of the Research Foundation – Flanders (FWO). Debby Mangelings and Bieke Dejaegher are post-doctoral fellows of the Research Foundation – Flanders (FWO). The authors also thank the bilateral project DWTC (03/V09).

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