Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1085 (2005) 230–239
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Development of a *Ginkgo biloba* fingerprint chromatogram with UV and evaporative light scattering detection and optimization of the evaporative light scattering detector operating conditions

A.M. van Nederkassel, V. Vijverman, D.L. Massart, Y. Vander Heyden *

Department of Analytical Chemistry and Pharmaceutical Technology, Pharmaceutical Institute, Vrije Universiteit Brussel-VUB, Laarbeeklaan 103, 1090 Brussels, Belgium

Received 9 February 2005; received in revised form 18 May 2005; accepted 27 May 2005

Abstract

A fingerprint chromatogram of a standardized *Ginkgo biloba* extract is developed on a monolithic silica column using a ternary gradient containing water, *iso*-propanol and tetrahydrofuran. For the detection, UV and evaporative light scattering (ELS) detectors are used, the latter allowing detection of the poor UV absorbing compounds as ginkgolides (A–C and J) and bilobalide in the extract. The complementary information between the UV and ELS fingerprint is evaluated. The ELS detector used in this study can operate in an impactor 'on' or 'off' mode. For each mode, the operating conditions such as the nebulizing gas flow rate, the drift tube temperature and the gain are optimized by use of three-level screening designs to obtain the best signal-to-noise (S/N) ratio in the final ELS fingerprint chromatogram. In both impactor modes, very similar S/N ratios are obtained for the nominal levels of the design. However, optimization of the operating conditions resulted, for both impactor modes, in a significant increase in S/N ratios compared to the initial evaluated conditions, obtained from the detector software.

Keywords: Fingerprint chromatogram; *Ginkgo biloba*; ELSD; Three-level screening design

1. Introduction

Phytopharmaceuticals containing the *Ginkgo biloba L.* extract are worldwide used to treat cardiovascular and cerebrovascular diseases such as Alzheimer's disease [1–3]. The beneficial effects are due to the presence of ginkgolides (A–C and J) and bilobalide that represent together with the flavonoids the active constituents [4]. Besides these, the extract contains many other components of which we have only limited knowledge, making the evaluation of its quality and safety very difficult [5]. Because of the complexity of the extract, a qualitative and quantitative analysis of each individual compound is almost impossible and therefore, fingerprint technology was introduced to achieve quality control of herbal medicines [5,6]. The idea is to develop a fingerprint chromatogram (or electropherogram) of the herbal medicine in which as many compounds as possible are separated, and to compare the fingerprint with that of a standardized extract in order to achieve authentication, identification and quality control of the herbal medicine [5,7,8].

Often, HPLC-UV is used for the development of fingerprints. However, compounds with very few chromophore groups, as for instance ginkgolides A–C and J and bilobalide in the *Ginkgo biloba* extract, will poorly absorb UV radiation and are therefore difficult to detect with this type of detectors [1,2,4,9]. The ELS detector is then an alternative detector [1,2] that can be connected in series with the UV detector. The operation of the ELS detector is based on a three-step process: mobile phase nebulization, evaporation and detection. It measures the amount of light scattered by sample components that do not evaporate during the mobile phase evaporation stage. Any compound having a lower volatility than the mobile phase is detected and the detector output reflects the quantity or mass of total analyte responsible for the light scattering [10–13]. The use of the

* Corresponding author. Tel.: +32 2 477 47 34; fax: +32 2 477 47 35.
E-mail address: yvanvdh@vub.ac.be (Y.V. Heyden).

0021-9673/$ – see front matter © 2005 Published by Elsevier B.V.
doi:10.1016/j.chroma.2005.05.110

ELS detector for the determination of ginkgolides in *Ginkgo biloba* leaf extracts was introduced by Camponovo et al. [14]. Today, several studies are published describing a HPLC-ELS method for the analysis of ginkgolides and bilobalide [1,2,8]. These methods allow determination of these compounds after extraction but do not provide information about other *Ginkgo biloba* extract constituents, which also contribute to the quality of the extract. Therefore, the goal of this paper is to develop a ELS fingerprint chromatogram for a standardized extract of the leaves of *G. biloba L.* Since the sensitivity of the ELS detector is known to change in solvent gradients, mainly due to changes in droplet size [13], a UV detector is placed before the ELS detector for maximal sensitivity. The information in, and the quality of the UV and ELS fingerprint chromatograms is compared. The UV–ELS fingerprint is developed on a monolithic silica column, which is chosen for its lower column backpressures and faster separations, compared to conventional columns [15,16]. In this study, two monolithic columns are coupled in series to increase the separation efficiency, necessary for the separation of this complex extract. Examples of the use of monolithic columns in pharmaceutical analysis can be found in [15–17]. The UV–ELS fingerprint developed in this study, is obtained with a ternary gradient elution method. After method development, the four ELSD operating conditions (the impactor mode, the nebulizing gas flow rate, the drift tube temperature and the gain) are optimized to reduce the signal-to-noise ratio in the ELS fingerprint. For that purpose, a three-level screening design is performed [18]. Finally, a methodology for quality control by use of the above-developed fingerprints is proposed.

2. Theory

2.1. Evaporative light scattering detector parameters to optimize

The ELS detector used in this study requires the optimization of four parameters: the impactor mode, the nebulizing gas flow rate (NGF), the drift tube temperature (DTT) and the gain. The impactor can be either in the on-mode, meaning that the teflon coated stainless steel plate (impactor) is placed perpendicular to the flow path of the aerosol through the drift tube, or in the off-mode if it is placed parallel [10]. In the latter case, it does not disturb the aerosol flow during its travel towards the detector cell, resulting into a maximal sensitivity. In the impactor on-mode, on the contrary, only small droplets pass around the impactor to reach the detector cell and large droplets will exit the system through the drain tube (see Fig. 1).

The nebulizing gas flow rate, the drift tube temperature and the gain are chosen as a function of the impactor mode. In the impactor off-mode, the NGF and DTT are higher to achieve adequate mobile phase evaporation [10]. They are chosen between 0 and 4.0 l/min, and between 25 and 120 °C, respectively. The gain controls the detector signal

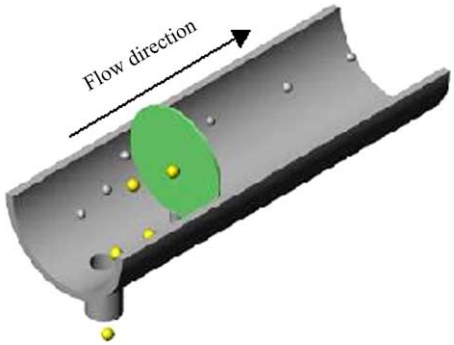

Fig. 1. Impactor of the ELSD in the on-mode. Figure adapted from ref. [19].

amplification to ensure the detection of small peaks. It can take values 1, 2, 4, 8 or 16. A gain of 1 produces an unamplified signal and each other value *n* produces a *n*-fold signal amplification compared to the original signal [10].

For inexperienced users it is not straightforward to define the above parameters and most users use initial parameter settings which can be computed with the ELSD software or by use of Table 1. In gradient elution methods, they are computed for the mobile phase composition which is the least volatile. However, these initial values need further optimization to obtain the best signal-to-noise ratio. Therefore, during method development, initial values derived from Table 1 are used, and for the final ELS fingerprint a screening design is performed to optimize these parameters.

2.2. Three-level screening design

For the optimization of the ELSD parameters, two three-level screening designs are performed, one for each impactor mode. In both screening designs, the influence of the NGF, the DTT and the gain is investigated on the S/N ratio of three peaks from the final ELS fingerprint. Since the three-level design allows to screen four factors, one imaginary factor (dummy) is included, allowing to estimate the experimental

Table 1
Initial drift tube temperature and nebulizing gas flow rate of the ELSD (in the impactor off-mode) for HPLC separations at 1.0 ml/min, using a standard 4.6 mm I.D. column, extracted from [10]

Solvent	Drift tube temperature (°C)	Gas flow rate (l/min)
Acetone	30	0.6
Acetonitrile	70	1.7
Chloroform	40	1.5
Heptane	50	1.5
Hexane	40	1.6
iso-Propanol	55	1.7
Methanol	60	1.6
Methylene chloride	50	1.6
Tetrahydrofuran	60	1.7
Water	115	3.2
Methanol:water (90:10)	75	2.0
Acetonitrile:water (75:25)	80	2.0

Table 2
Three-level screening design for four factors

Experiment	Factors			
	Drift tube temperature (°C)	Gas flow rate (l/min)	Gain	Dummy
Nominal	0	0	0	0
2	0	−	0	+
3	0	0	−	0
8	0	+	+	−
Nominal	0	0	0	0
1	−	0	+	+
5	−	+	0	0
9	−	−	−	−
Nominal	0	0	0	0
4	+	0	0	−
6	+	−	+	0
7	+	+	−	+
Nominal	0	0	0	0

Four nominal experiments are included in the set-up. Experiments are sorted by drift tube temperature.

error [20]. Each factor is tested at three levels (level (0), the nominal or central level, and levels (−) and (+), the extreme levels). The design contains nine experiments and is well-balanced meaning that none of the main factor effects is confounded [18]. Before and after the design and after each third experiment of the design a nominal experiment is performed, i.e. an experiment where all factors are at level 0. The latter experiment is used to check for drift (time effects) during the execution of the design and to evaluate the repeatability of the fingerprint. For experimental convenience, the design experiments are sorted by drift tube temperature because adjusting this factor requires a long equilibration of the ELS detector. The design experiments are shown in Table 2. The effect of the examined factors on the S/N ratios of the three peaks is computed [18] and a *t*-test [20] is performed to evaluate whether the effect is significant. A factor is concluded to have a significant effect if the absolute value of its effect is larger than the critical effect [20]. The algorithm of Dong is applied to identify significant effects [20,21].

2.3. Measured response

The response measured, is the signal-to-noise (S/N) ratio of three peaks, computed with the equation of the European Pharmacopoeia [22]

$$\mathrm{S/N} = \frac{2H}{h_\mathrm{n}} \quad (1)$$

where H is the peak height and h_n is the absolute value of the largest baseline noise amplitude in the blank chromatogram around the time where the peak appears in the *Ginkgo biloba* fingerprint. However, in this study the noise parameter h_n was not derived from a blank injection as prescribed by the European Pharmacopoeia, but from the baseline between 52 and 60 min of the *Ginkgo biloba* ELS fingerprint.

3. Experimental

3.1. Instruments, chemicals and mobile phases

3.1.1. Instruments

The high performance liquid chromatography (HPLC) system consists of a L-7100 pump, L-7612 solvent degasser, L-7250 autosampler, L-7360 column oven, L-7400 UV-detector and a D-7000 interface from Merck-Hitachi (Tokyo, Japan). This system is operated with the LaChrom D-7000 HPLC System Manager software (Merck-Hitachi). An Alltech 2000 ELS detector (Alltech Associates, Deerfield, USA) is also connected to the chromatograph, with pressurized air (60 psi, 4.14 bar) as nebulizing gas. It is operated with the ELSD 2000 Control software (Alltech).

The column temperature is kept constant at 35 °C, the injection volume and mobile phase flow rate are 10 μl and 1 ml/min, respectively. The UV detector measured absorbance at 267 nm.

3.1.2. Chemicals

Ethanol (pro analyse quality) from Merck (Darmstadt, Germany) is used to dissolve the *Ginkgo biloba* extract.

3.1.3. Mobile phases

The mobile phases for the separation of the *Ginkgo biloba* extract are prepared with methanol (MeOH), acetonitrile (ACN) (both HPLC grade quality) from Fisher Scientific (Leicestershire, UK), *iso*-propanol (*i*-PrOH) or tetrahydrofuran (THF) (both HPLC gradient quality from Merck) on the one hand and Milli-Q water (obtained with the Milli-Q water purification system from Millipore, Molsheim, France) containing 0.05% trifluoroacetic acid (TFA) from Sigma–Aldrich (Steinheim, Germany) on the other.

3.1.4. Column

Two Chromolith Performance (100 mm × 4.6 mm) columns both RP-18e from Merck, which are coupled in series with a column coupler (Merck), are used. A Chromolith guard column RP-18e (5 mm × 4.6 mm, Merck) was placed before the analytical ones.

3.2. Preparation of the Ginkgo biloba extract and standards

The *Ginkgo biloba* dry extract (GBE 20030106) was provided by the TSI Natural products company (Xuzhou, China). The extract solution is prepared as follows: 3.0026 g of dry extract was dissolved in 50.0 ml 80/20 (v/v) ethanol/water on a Branson 5210 ultrasonic bath (Branson Ultrasonic Corporation, Danbury, USA) and then filtered through a 0.2 μm membrane filter (Pall Gelman Laboratory, Karlstein, Germany). The extract solution was stored in dark glass recipients at 8 °C. Standards of ginkgolides A (1.42 mg/ml), B (2.24 mg/ml) and C (1.97 mg/ml) from Fluka

Chemie (Buchs, Switzerland) and bilobalide (2.20 mg/ml) from Sigma–Aldrich Chemie were prepared in methanol.

4. Results and discussion

4.1. Development of a Ginkgo biloba fingerprint chromatogram

The UV–ELS fingerprint chromatogram for the *G. biloba L.* extract is developed on a stationary phase consisting of a Chromolith guard column and two Chromolith Performance columns, coupled in series. During method development, the mobile phase flow rate is kept constant at 1 ml/min, the column temperature at 35 °C and the injection volume at 10 μl. The UV detection wavelength is set at 267 nm, which is the experimental λ_{max} of the *Ginkgo biloba* extract in organic solvents as MeOH and ACN. The ELS detector is operated in the impactor off-mode for maximal sensitivity. The gain parameter is set at 4, the maximum gain for which the peaks could be measured within scale. The other two ELSD parameters (the drift tube temperature and nebulizing gas flow rate) are always adapted according to the least volatile mobile phase composition of the gradient. For each gradient, they are determined with the ELSD software, or by use of Table 1. From Table 1, it follows that for instance, for a 5/95 (v/v) MeOH/water ratio, one obtains a DTT equal to 112.25 °C (60 × 5% + 115 × 95%) and a NGF of 3.12 l/min (1.6 × 5% + 3.2 × 95%). These ELSD conditions are initial conditions and only for the final ELS fingerprint, the ELSD conditions will be optimized.

The methodology to develop the UV–ELS fingerprint is as follows. First simple binary gradients consisting of water (with 0.05% TFA) and either methanol, acetonitrile, *iso*-propanol or tetrahydrofuran are tested. The percentage organic modifier is increased from 5 to 95% (vol.%) within 50 min and is kept constant at 95% till 60 min. Before injection, the columns are equilibrated during 25 min with the 5% organic modifier mobile phase. From the obtained chromatograms, the percentages organic modifier at the elution of the first and the last peak is estimated for each binary gradient. These conditions will be used in the following run. The new start condition is the mobile phase composition at which the first peak elutes. The new end condition is the mobile phase composition at 5 min before the last peak elutes. The four optimized binary gradients, obtained in this way, are (i) 25–50% MeOH, (ii) 10–25% ACN, (iii) 5–20% *i*-PrOH and (iv) 5–40% THF, all vol.%. The end of the gradient is reached after 50 min and these conditions are kept constant till 60 min. The UV–ELS fingerprints obtained for these binary gradients are shown in Fig. 2. The ELSD operating conditions, used for each gradient, are summarized in Table 3. Two criteria, the number of peaks in the UV and ELS signal, and the number of additional peaks in the ELS signal evaluate the quality of the fingerprints, obtained with these four gradients. In the UV fingerprints, respectively 52, 75, 63 and 72 peaks are detected while in the ELS fingerprints, respectively 42, 61, 48 and 59 peaks are seen. The MeOH gradient results in a fingerprint

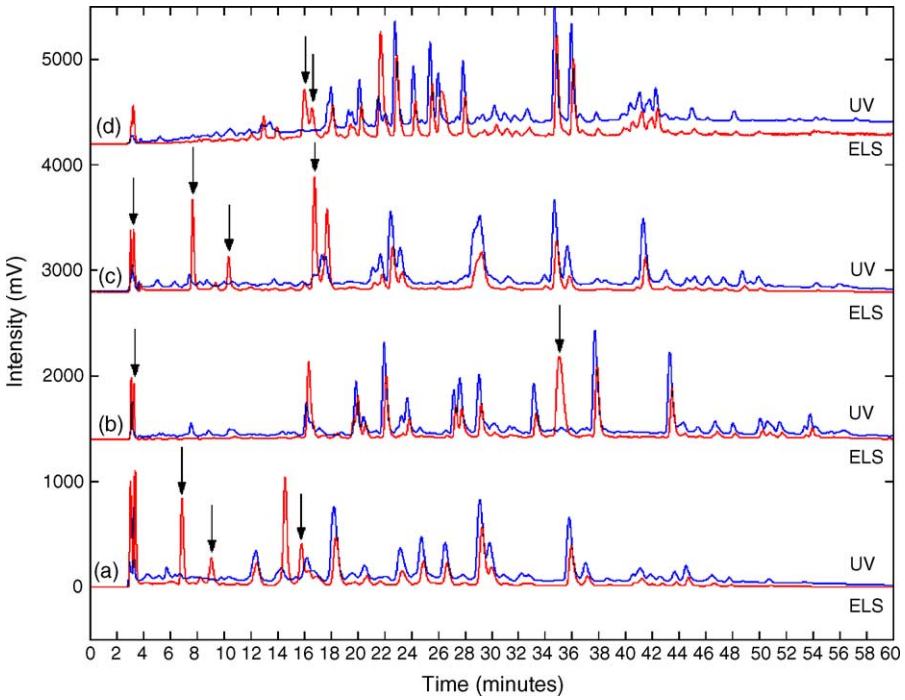

Fig. 2. HPLC-UV–ELS profiles of a *Ginkgo biloba* extract (60.05 g/l) separated on a monolithic silica column with a binary gradient of (a) 25–50% MeOH, (b) 10–25% ACN, (c) 5–20% *i*-PrOH and (d) 5–40% THF. The ELSD operating conditions in impactor off-mode are given in Table 3. The arrows indicate additional peaks seen in the ELS profile.

Table 3
Initial values for the drift tube temperature and the nebulizing gas flow rate computed with the ELSD software for each binary gradient, consisting of Milli-Q water (+0.05% TFA) and organic modifier

Gradient (vol.%)	Drift tube temperature (°C)	Gas flow rate (l/min)
5–95% MeOH	112.3	3.1
5–95% ACN	112.8	3.1
5–95% *i*-PrOH	112.0	3.1
5–95% THF	112.3	3.1
25–50% MeOH	101.3	2.8
10–25% ACN	110.5	3.1
5–20% *i*-PrOH	112.0	3.1
5–40% THF	112.3	3.1

The end concentration of the gradient is reached after 50 min and is kept constant till 60 min.

with the lowest number of peaks in the UV and ELS signal. The highest number of peaks is obtained with the ACN and THF gradients. From Fig. 2, it can be seen that the ELS profile always reveals fewer and smaller peaks than the UV profile. The higher detection limit of the ELS detector might explain this [11], but also the high drift tube temperature, used in the impactor off-mode, can be the reason. Extract components might evaporate and will not be seen in the ELS signal. However, the ELS signal has the advantage having a flatter baseline, while in the UV chromatogram baseline drift can occur due to mobile phase UV absorption. The baseline drift is most pronounced in the UV fingerprint obtained for the THF gradient (see Fig. 2d). Besides the total number of peaks, seen in the UV and ELS fingerprints, also the number of additional peaks in the ELS signal is important since the *Ginkgo biloba* extract contains ginkgolides and bilobalide with low UV absorbance. While the ELS signals of the MeOH, ACN and THF gradients reveal only two or three additional peaks, four additional peaks are obtained with the *iso*-propanol gradient. The additional peaks are indicated with an arrow in Fig. 2. Since both criteria, the total number of peaks and the number of additional peaks in the ELS signal, are important quality criteria for fingerprint development, a ternary gradient is developed using *iso*-propanol and tetrahydrofuran as organic modifiers. *iso*-Propanol was selected since it reveals four additional peaks in its ELS fingerprint (Fig. 2c) and tetrahydrofuran since the highest number and the narrowest peaks are obtained for this organic modifier (see Fig. 2d). From Figs. 2c and d, it can be seen that the additional peaks for the *iso*-propanol gradient elute within the first 18 min, while for the tetrahydrofuran gradient the majority of the peaks elute after 18 min. A different selectivity between both systems is observed. For this reason, a ternary gradient of water/*iso*-propanol/tetrahydrofuran was composed, starting with *iso*-propanol as organic modifier and increasing the tetrahydrofuran fraction during the gradient. The gradient is shown in Table 4. The UV and ELS fingerprints obtained with this ternary gradient, reveal 77 and 73 peaks, respectively, and are shown in Fig. 3. These fingerprints separate more peaks than those obtained for the binary gradients. Moreover, seven additional peaks can be seen in the ELS fingerprint. They are indicated with a full arrow in Fig. 3. Some of these additional peaks were identified, as for instance the ginkgolides

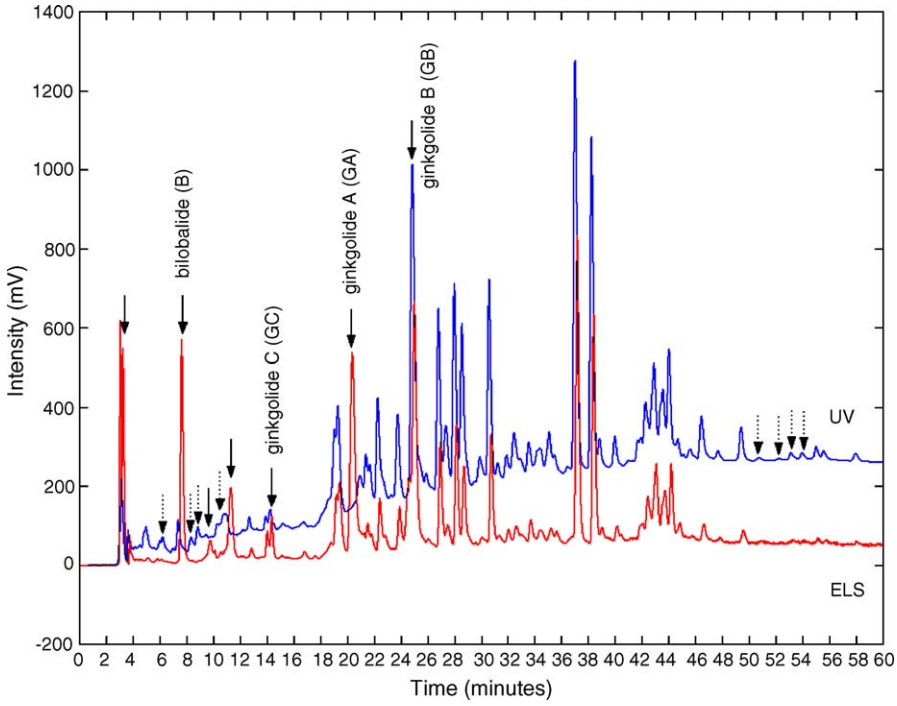

Fig. 3. HPLC-UV–ELS profiles of a *Ginkgo biloba* extract (60.05 g/l) separated on a monolithic silica column with a *i*-PrOH/THF/water + 0.05%TFA gradient (see Table 4). ELSD operating conditions: impactor off-mode, DTT = 112.0 °C, NGF = 3.1 l/min and gain = 4. Full arrows indicate additional peaks seen in the ELS profile, dotted arrows indicate peaks only seen in the UV profile.

Table 4
Ternary gradient of the final *Ginkgo biloba* fingerpint

Time (min)	*iso*-Propanol (%)	Tetrahydrofuran (%)	Milli-Q + 0.05% TFA (%)
0	5	0	95
15	0	13	87
50	0	40	60
60	0	40	60

A–C and the bilobalide peak. The UV fingerprint also reveals peaks which could not be detected in the ELS fingerprint. These peaks are indicated with a dotted arrow, for instance the peaks at 6.2, 8.4, 8.7, 10.7, 50.8, 52.2, 53.1 and 53.9 min (Fig. 3). From this, it can be concluded that the UV and ELS fingerprints contain complementary information. It is also seen that the obtained ELS signal is rather noisy between 45 and 60 min. In the next section it is therefore examined whether the S/N ratio in this ELS fingerprint chromatogram can be increased by optimization of the by the ELSD software proposed input parameters. Furthermore, it is examined whether operating the ELS detector in the impactor on-mode results in better S/N ratios than in the impactor off-mode.

4.2. Optimization of the ELSD parameters

4.2.1. Design for the ELSD in impactor off-mode

The ELS fingerprint developed in Section 4.1. Fig. 3 is obtained with the ELS detector operating in the impactor off-mode. The drift tube temperature, nebulizing gas flow rate and gain setting were 112 °C, 3.1 l/min and 4, respectively. These operating conditions are now used as nominal levels in the impactor-off design, except from the gain, for which the nominal level is chosen to be 2, which is one value lower than the highest value for which before the peaks were still within scale. The (−) and (+) levels are chosen as ±5.0 °C (DTT), ±0.5 l/min (NGF) and ± one gain setting. Table 5 gives an overview of the examined factors and their levels.

Table 5
Low (−), nominal (0) and high (+) levels used in the three-level screening designs for the impactor on- and off-modes

	Factors	(−) level	(0) level	(+) level
Impactor off				
A	Temperature (°C)	107	112	117
B	Gas flow rate (l/min)	2.6	3.1	3.6
C	Gain	1	2	4
Impactor on				
A	Temperature (°C)	40	55	70
B	Gas flow rate (l/min)	0.5	1.5	2.5
C	Gain	4	8	16

The ELSD conditions proposed by the ELSD software are underlined.

Three responses are measured, being the signal-to-noise ratios of the peaks with retention time 7.6, 19.0 and 37.1 min, respectively (Fig. 4). The first two peaks were identified as bilobalide and ginkgolide A by use of standards. The peaks are selected because they are important components in the extract and are situated at different locations in the

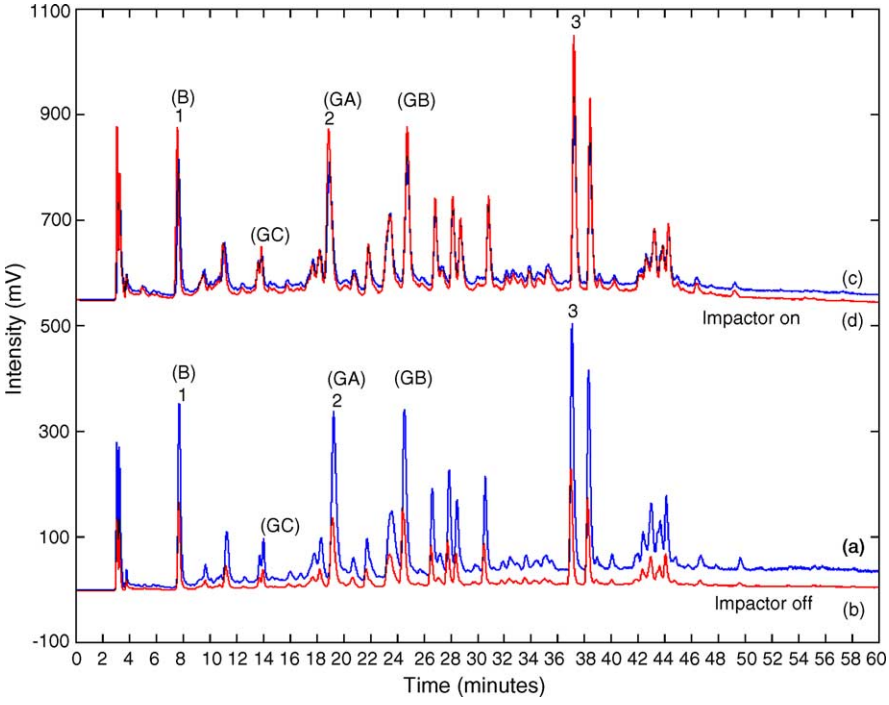

Fig. 4. HPLC-ELS profiles of a *Ginkgo biloba* extract (60.05 g/l) separated on a monolithic silica column with a *i*-PrOH/THF/water + 0.05%TFA gradient (see Table 4) before (a and c) and after (b and d) optimization of the ELSD operating conditions in the impactor off- and on-modes, respectively. ELSD operating conditions: (a) (nominal conditions): DTT = 112 °C, NGF = 3.1 l/min, gain = 2, (b) (conditions of experiment 7): DTT = 117 °C, NGF = 3.6 l/min, gain = 1, (c) (nominal conditions): DTT = 55 °C, NGF = 1.5 l/min, gain = 8, (d) (conditions of experiment 8): DTT = 55 °C, NGF = 2.5 l/min, gain = 16.

chromatogram. For experiments 4 and 7 of the design (where the drift tube temperature is at high level (see Table 2) the highest S/N ratios are obtained (Table 6). At higher temperatures, the aerosol droplets are obviously evaporated better. The S/N ratios for the first nominal level were found surprisingly high compared to those in experiment 4. However, this is probably due to experimental errors since this was not seen for the following nominal levels. Experiments 1 and 9 result in the lowest S/N ratios, which is because the drift tube temperature is at the (−) level. At low temperature, small mobile phase droplets might remain after the evaporation step, resulting in a higher noise level. When the ELS fingerprint chromatograms obtained for the nominal experiment (Fig. 4a) and experiment 7 (Fig. 4b) are compared, it is seen that in the latter a signal with less noise and baseline drift is obtained. The improvement in the quality of the ELS fingerprint is clearly observed.

The factor effects computed for the three peaks are shown in Table 7. From this Table, it can be seen that the effect of changing the temperature from the (−) level to the (0) level and from the (−) level to the (+) level is significant at the 5% level for the three peaks. The effect is positive meaning that higher S/N ratios are obtained when the temperature of the drift tube is at higher level. For the nebulizing gas flow rate a positive effect on the S/N ratio, although not significant, is found. For the gain no significant effect is seen. It can be concluded that when the temperature is chosen 5 °C higher than the nominal settings, clearly higher S/N ratios are found. Gas flow changes of ±0.5 l/min and changing the gain setting into 1 higher or lower value does not statistic significantly influence the S/N ratio. However, the latter factors do influence the S/N ratios as can be seen by comparing the responses for experiments 4 and 7 (Table 6). In experiment 7, higher S/N ratios are found because the gas flow rate is at high level and the gain at low. It can be concluded that the optimal ELSD parameters in the impactor off-mode are DTT = 117 °C, NGF = 3.6 l/min and a gain = 1 (see Fig. 4b).

Table 6
Responses (S/N ratios) measured for each experiment of the screening design with the ELSD in the impactor off-mode

Experiment	Peak 1	Peak 2	Peak 3
Nominal	67.1	62.2	88.2
2	31.7	32.2	44.1
3	45.8	41.2	59.9
8	62.7	59.0	86.3
Nominal	48.7	48.0	72.3
1	15.4	13.7	24.6
5	21.8	20.9	31.5
9	7.5	7.9	16.4
Nominal	53.8	48.4	71.9
4	69.3	54.5	88.0
6	58.9	51.4	76.9
7	80.2	63.1	106.5
Nominal	51.7	44.8	72.2

Peaks 1–3 are the peaks with retention time 7.6, 19.0 and 37.1 min in Fig. 4.

Table 7
Effects of the examined factors on the S/N ratio of peaks 1–3 (see Fig. 4) for the ELSD in the impactor off-mode

Factors	$E_{-,0}$	$E_{0,+}$	$E_{-,+}$
	Peak 1 ($E_{\text{critical 5\%}}$ = 25.67)		
(A) Temperature (°C)	**31.81**	22.73	**54.54**
(B) Gas flow rate (l/min)	10.79	11.40	22.19
(C) Gain	−3.56	4.74	1.17
(D) Dummy	−4.32	0.27	−4.04
	Peak 2 ($E_{\text{critical 5\%}}$ = 18.34)		
(A) Temperature (°C)	**29.97**	12.19	**42.16**
(B) Gas flow rate (l/min)	5.93	11.23	17.15
(C) Gain	−1.53	5.47	3.94
(D) Dummy	−2.65	−1.46	−4.11
	Peak 3 ($E_{\text{critical 5\%}}$ = 33.03)		
(A) Temperature (°C)	**39.27**	27.01	**66.27**
(B) Gas flow rate (l/min)	11.67	17.27	28.94
(C) Gain	−6.41	8.10	1.68
(D) Dummy	−7.46	2.30	−5.15

The significant effects are bold and underlined.

4.2.2. Design for the ELSD in impactor on-mode

Although the *G. biloba L.* extract is not expected to contain volatile compounds and thus the use of the impactor off-mode is preferred, it is examined whether better responses can be obtained by operating the ELSD in the impactor on-mode. The initial operating conditions in the impactor on-mode, proposed by the ELSD software or manual, are a drift tube temperature of 40 °C and a gas flow rate of 1.5 l/min for separations with highly aqueous mobile phases and/or steep gradients at a mobile phase flow rate of 1 ml/min. Because of the shielding effect of the impactor on the detection cell, these values are fixed and irrespective of the mobile phase composition. Preliminary experiments showed that the drift tube temperature and the nebulizing gas flow rate may not be chosen below 40 °C and 0.5 l/min, respectively, for adequate mobile phase evaporation. Therefore, these values were selected as the respectively (−) levels. The highest gain setting for which the peaks still fall within the scale is equal to 16 and is chosen as (+) level. Taking into account these minimum and maximum values, the nominal levels of the drift tube temperature, nebulizing gas flow rate and gain are chosen equal to 55 °C, 1.5 l/min and 8, respectively. The extreme levels for the first two parameters are the nominal value ±15.0 °C and ±1.0 l/min, respectively. The (−) and (+) level for the gain is 4 and 16, respectively (see Table 5).

After performing the design experiments, the S/N ratios are measured and the effects are computed. The highest S/N ratios were found for experiments 7 and 8, where the gas flow rate is at (+) level, while the lowest S/N ratios were found for experiments 2 and 9 which are performed at the (−) level gas flow rate (see Tables 2 and 8). When a chromatogram of the nominal level (Fig. 4c) is compared to that of experiment 8 (Fig. 4d), it is seen that the noise and baseline drift is reduced for the conditions in experiment 8.

Table 8
Responses (S/N ratios) measured for each experiment of the screening design with the ELSD in the impactor on-mode

Experiment	Peak 1	Peak 2	Peak 3
Nominal	53.9	47.7	71.0
2	13.8	25.8	37.3
3	73.9	66.5	100.8
8	73.1	69.0	109.5
Nominal	53.3	49.1	73.5
1	42.2	37.6	62.4
5	44.3	36.0	52.6
9	11.2	27.3	43.1
Nominal	–	–	–
4	51.0	42.7	68.8
6	19.6	26.2	50.6
7	76.9	67.9	101.5
Nominal	48.6	44.9	69.5

No responses for the third nominal level due to data loss. Peaks 1–3 are the peaks with retention time 7.6, 19.0 and 37.1 min in Fig. 4.

Table 9 shows the effects of changes in the three factors. From this Table, it can be seen that the changes in the nebulizing gas flow rate have the highest effect on the S/N ratio. Changing the gas flow rate from 0.5 to 1.5 or 2.5 l/min results in clearly higher S/N ratios for peaks 2 and 3 and a statistic significantly higher S/N ratio for peak 1. From Table 9, it can also be concluded that keeping the temperature at the 0 level (55 °C) results in the highest S/N ratio for the three peaks. It can thus be concluded that the initial drift tube temperature of 40 °C (=(−) level), proposed by the user manual, is not found the optimal value. The changes in gain were not found to have a statistic significant effect. However, from Table 9 it follows that low gain levels would result in higher S/N ratios. From this, it can be concluded that the optimal ELSD settings for the impactor on-mode are DTT = 55 °C, NGF = 2.5 l/min and a gain of 4. Notice that our design does not contain an experiment under these conditions (see Table 2) and thus no such chromatogram can be shown.

Table 9
Effects of the examined factors on the S/N ratio of peaks 1–3 (see Fig. 4) for the ELSD in impactor on-mode

Factors	$E_{-,0}$	$E_{0,+}$	$E_{-,+}$
	Peak 1 ($E_{\text{critical 5\%}}$ = 25.34)		
(A) Temperature (°C)	21.07	−4.46	16.61
(B) Gas flow rate (l/min)	**40.85**	9.07	**49.92**
(C) Gain	−17.66	8.63	−9.03
(D) Dummy	0.85	−1.62	−0.77
	Peak 2 ($E_{\text{critical 5\%}}$ = 32.93)		
(A) Temperature (°C)	20.18	−8.20	11.97
(B) Gas flow rate (l/min)	22.52	8.71	31.23
(C) Gain	−19.06	9.45	−9.61
(D) Dummy	−3.42	0.89	−2.54
	Peak 3 ($E_{\text{critical 5\%}}$ = 48.85)		
(A) Temperature (°C)	29.83	−8.89	20.94
(B) Gas flow rate (l/min)	33.66	10.54	44.20
(C) Gain	−28.91	21.26	−7.65
(D) Dummy	−5.82	−0.95	−6.77

The significant effects are bold and underlined.

Comparing the results of both designs, it is seen that for the impactor off-mode, the drift tube temperature is the critical parameter, while for the impactor on-mode the nebulizing gas flow rate is more important. From the fingerprints obtained for the optimized ELSD parameters in the impactor off- (Fig. 4b) and on-mode (Fig. 4d), it is seen that a better sensitivity (more and higher peaks) is found for the fingerprint recorded in the impactor on-mode. However, this signal shows more baseline drift.

4.3. Fingerprint repeatability

The day-to-day repeatability was tested by repeating the separation of the *Ginkgo biloba* extract at nominal level (nominal design experiments) for both impactor modes. Fig. 5 shows four ELS fingerprints obtained in the impactor off-mode; two recorded on day one (Fig. 5a and b) and two on the following day (Fig. 5c and d). The number of peaks and the separation is repeatable, which is the main interest when developing fingerprints. The day-to-day repeatability of the ELS fingerprints in the impactor on-mode was also found to be good (fingerprints not shown).

4.4. Quality control of Ginkgo biloba extracts by use of UV and ELS fingerprints

Below we propose a methodology to estimate the quality of *Ginkgo biloba* extracts by chemometric analysis of their UV and ELS fingerprints. In order to allow such chemometrical analysis, the fingerprints should be warped in order to align corresponding peaks. Alignment methods, which do not require peak detection and identification, as for instance, correlation optimized warping (COW) [23], parametric time warping (PTW) [24] or semi-parametric time warping (STW) [25], can be used [26]. Chemometric techniques such as principal component analysis [27] or projection pursuit [27] allow visualization of the variance between *Ginkgo biloba* fingerprints from different origin, based on differences in their peak profile. When standardized extracts are included in the data analysis, one can use the distance between the objects of the standardized extracts and of the extracts under evaluation to estimate how different their fingerprints are. Extracts with large distance from the standardized extracts have a more dissimilar fingerprint and most likely also their quality is different. Therefore, the quality of such extracts is considered unacceptable unless the difference in their fingerprints can be explained. Initially, we would suggest to use both UV and ELS fingerprints for chemometrical analysis to explore their complementary information. The presence of ginkgolide peaks in the ELS profiles may reveal to what extend the ginkgolides contribute to the quality of the *Ginkgo biloba* extracts. When, on the other hand, the quality of an extract can be quantified, it can be modeled

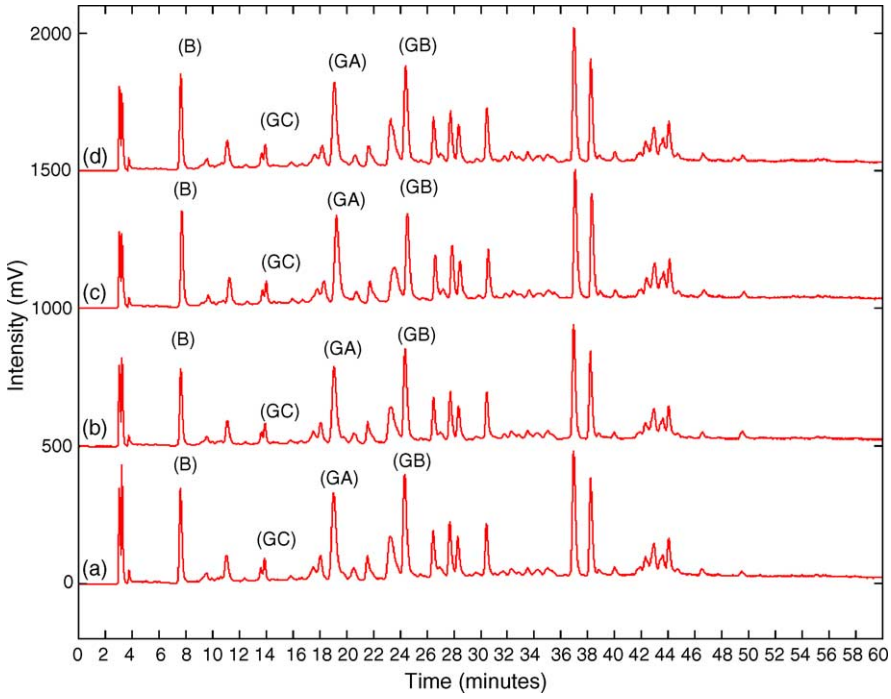

Fig. 5. HPLC-ELS profiles of a *Ginkgo biloba* extract (60.05 g/l) separated on a monolithic silica column with a *i*-PrOH/THF/water + 0.05%TFA gradient (see Table 4), injections repeated on day 1 (a and b) and day 2 (c and d). ELSD operating conditions in impactor off-mode: DTT = 112 °C, NGF = 3.1 l/min, gain = 2 (nominal conditions).

as a function of the fingerprint variables using multivariate calibration methods such as partial least squares regression (PLS) or principal component regression (PCR) [26].

5. Conclusions

A fingerprint chromatogram for the *Ginkgo biloba* extract is developed on a monolithic silica column, using a UV and ELS detector as operating detectors. A ternary gradient of *iso*-propanol, tetrahydrofuran and water was applied. The UV and ELS signals contain complementary information; the UV signal reveals volatile compounds or compounds below the detection limit of the ELSD, while the latter detector reveals non- and poorly UV absorbing compounds. The S/N ratios in the ELS fingerprint chromatograms were found very similar for both impactor modes when the nominal design levels for the ELSD operating conditions are used, obtained from the detector software. However, a significant increase in S/N ratios was found after optimization of these parameters. For our separation, it was found that a temperature increase of 5 °C (impactor off) or 15 °C (impactor on) and a gas flow rate increase of 0.5 l/min (impactor off) or 1 l/min (impactor on) result in clearly improved S/N ratios compared to the S/N ratios found when applying the ELSD operating conditions computed with the ELSD software. Since both the UV and ELS detector are used in this study, the ELSD can be operated in the impactor off-mode without the risk not detecting slightly volatile or low concentrated compounds, which are still UV absorbing. However, if only a ELS detector would be used, the impactor on-mode is advised for a better detection of all components, in spite of the stronger baseline drift.

Acknowledgement

A.M van Nederkassel is grateful to the Fund for Scientific Research (FWO), Flanders for financial support.

References

[1] C. Tang, X. Wei, C. Yin, J. Pharm. Biomed. Anal. 33 (2003) 811.
[2] W. Li, J.F. Fitzloff, J. Pharm. Biomed. Anal. 30 (2002) 67.
[3] S. Bratman, A.M. Girman, Handbook of Herbs and Supplements and their Therapeutic Uses, Mosby, St. Louis, 2003, pp. 593–601.
[4] A.G. Jensen, K. Ndjoko, J. Wolfender, K. Hostettmann, F. Camponovo, F. Soldati, Phytochem. Anal. 13 (2002) 31.
[5] F. Gong, Y. Liang, P. Xie, F. Chau, J. Chromatogr. A 1002 (2003) 25.
[6] WHO, Guidelines for the Assessment of Herbal Medicine, Munich, World Health Organization, 1991.
[7] Y. Ji, Q. Xu, Y. Hu, Y. Vander Heyden, J. Chromatogr. A 1066 (2005) 97.
[8] T.A. van Beek, J. Chromatogr. A 967 (2002) 21.
[9] T.A. van Beek, H.A. Scheeren, T. Rantio, W.Ch. Melger, G.P. Lelyveld, J. Chromatogr. 543 (1991) 375.
[10] Manual of the Alltech ELSD 2000 Evaporative Light Scattering Detector, Alltech Associates, Deerfield, USA, March 2003.
[11] C.S. Young, J.W. Dolan, LC-GC 16 (2003) 2.
[12] R.P.W. Scott, Chromatographic Detectors, Chromatographic Science Series, vol. 73, Marcel Dekker, New York, 1996, pp. 211–222.
[13] D. Parriott, A Practical Guide to HPLC Detection, Academic Press Inc., San Diego, 1993, pp. 256–261.

[14] F.F. Camponovo, J.-L. Wolfender, M.P. Maillard, O. Potterat, K. Hostettmann, Phytochem. Anal. 6 (1995) 141.
[15] K. Cabrera, D. Lubda, H. Eggenweiler, H. Minakuchi, K. Nakanishi, J. High Resol. Chromatogr. 23 (1) (2000) 93.
[16] D. Lubda, K. Cabrera, W. Kraas, C. Schaefer, D. Cunningham, LC-GC Europe 14 (2001) 730.
[17] A.M. van Nederkassel, A. Aerts, A. Dierick, D.L. Massart, Y. Vander Heyden, J. Pharm. Biomed. Anal. 32 (2003) 233.
[18] Y. Vander Heyden, M.S. Khots, D.L. Massart, Anal. Chim. Acta 276 (1993) 189.
[19] Website, A Closer Look at ELSD Technology, Softa Corporation, Chrom Tech Inc., 2004: http://www.chromtech.com/online_catalog/instruments/detectors/softa%20brochure.pdf.
[20] Y. Vander Heyden, A. Nijhuis, J. Smeyers-Verbeke, B.G.M. Vandeginste, D.L. Massart, J. Pharm. Biomed. Anal. 24 (2001) 723.
[21] F. Dong, Stat. Sinica 3 (1993) 209.
[22] The European Pharmacopoeia, fourth ed., Council of Europe, Strasbourg, France, 2003.
[23] N.P. Vest Nielsen, J.M. Carstensen, J. Smedsgaard, J. Chromatogr. A 805 (1998) 17.
[24] P.H.C. Eilers, Anal. Chem. 76 (2004) 404.
[25] P.H.C. Eilers, Semi-parametric Time Warping, submitted for publication.
[26] A.M. van Nederkassel, M. Daszykowski, D.L. Massart, Y. Vander Heyden, Prediction of total green tea antioxidant capacity from chromatograms by multivariate modeling, J. Chromatogr. A, in press.
[27] D.L. Massart, B.G.M. Vandeginste, L.M.C. Buydens, S. de Jong, P.J. Lewi, J. Smeyers-Verbeke, Data Handling in Science and Technology 20 A, Handbook of Chemometrics and Qualimetrics: Part A, 519, Elsevier, Amsterdam, The Netherlands, 1997, p. 569.