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Alternative solvents can make preparative liquid chromatography greener

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Abstract

To make preparative Reversed-Phase High Performance Liquid Chromatography (RP-pHPLC) greener, alternative solvents were considered among others in terms of toxicity, cost, safety, workability, chromatographic selectivity and elution strength. The less toxic solvents ethanol, acetone and ethyl acetate were proposed as possible greener replacements for methanol, acetonitrile and tetrahydrofuran (THF). For testing their feasibility, five Ginkgo terpene trilactones were used as model analytes. The best "traditional" eluent, i.e., methanol-THF-water (2:1:7) was used as benchmark. A generic two-step chromatographic optimization procedure by UHPLC consisting of (1) a simplex design using the Snyder solvent triangle and (2) HPLC modelling software was used. In the first step, two ternary mixtures were found (acetone-ethyl acetate-water (20.25:3.75:76) and ethanol-ethyl acetate-water (9.5:7.5:83)), which already gave better results than the benchmark. The second step in which the influence of the gradient time, temperature and ratio of the two best ternary isocratic solvents was studied, led to an optimal 10.5 min gradient and a minimum resolution of 5.76. In the final step, scale-up from 2.1 to 22 mm i.d. pHPLC columns proceeded successfully. When 0.5 g of sample was injected, baseline separation was maintained. Chromatographic and absolute purities for products exceeded 99.5% and 95% respectively. This example shows that using less toxic and cheaper solvents for pHPLC can go hand in hand with higher productivity and less waste.

Introduction

As part of a global drive towards sustainability, during the last 15 years much has been done on making chemistry, including analytical chemistry greener. To assess whether analyses are more or less green, one needs to take a comprehensive view as there are so many aspects to reckon with: worker health, process safety, environmental impact, sustainability, energy requirements, life cycle analysis (LCA), efficiency, regulations, amounts of solvents and chemicals needed, waste disposal and last but not least, costs. The buzz words in several reviews to achieve this are: smaller sample sizes, solvent-free extraction, simpler and more selective sample preparation, miniaturization, more benign solvents and greener chromatography.[1-8] To make phytochemical analysis greener, we have developed a solvent-free assay for the neurotoxin in Japanese star anise,[9] on-chip sample preparation for alkaloid extracts using only μL of solvents[10] and highly selective and energy-efficient magnetic nanoparticles replacing a column chromatographic step with halogenated solvents during the large-scale purification of Ginkgo allergens.[11]

In most cases however, an HPLC step is involved in the quantitation of non-volatile analytes and is not so easily replaced although Capillary Electrophoresis (CE) is sometimes a fine but somewhat less rugged alternative. As Gaber et al. estimated the total amount of waste generated by HPLC instruments worldwide at 34 million liters/year, even an incremental improvement would lead to a significant reduction of waste.[12] Recycling is difficult because most runs are gradient runs, and additionally there are safety, reproducibility, regulatory and manpower aspects to consider. In making HPLC greener, most can be gained in terms of solvents needed, by going to smaller internal diameters. In this respect nanoLC is the best (< 1

µL/min) but requires expertise and again is less rugged and therefore not popular. Nowadays 2.1 mm i.d. columns (80% reduction of solvent usage relative to traditional 4.6 mm i.d. columns) offer the best compromise between reproducibility and solvent usage. UHPLC allows the use of more efficient and thus shorter sub 2 µm columns and many protocols now make use of 5 cm columns leading to faster analyses and reduced solvent consumption.

The two most benign solvents for chromatography are water and carbon dioxide. There are several possibilities to increase the percentage of water in the mobile phase at the expense of organic modifiers. An example is superheated (or subcritical) water chromatography at temperatures of 100 to 250 °C.[13] For some analytes, 60 or 80 °C sufficed to use pure water as eluent.[14] Problems are stationary phase stability, analyte degradation and non-polar analytes. Other approaches include less retentive stationary phases[15, 16] or the addition of surfactants (Micellar Liquid Chromatography, MLC).[8, 17-21] In some cases, the latter technique works without the addition of any organic solvent. MLC is less suitable for highly non-polar analytes and unsuitable for preparative HPLC (pHPLC) because of the additives needed. The use of carbon dioxide requires the use of specialized Supercritical Fluid Chromatography (SFC) equipment and usually the addition of 10-30% of an organic modifier, like methanol or ethanol. It is efficient, especially for enantioselective separations[22] but less suitable for highly polar analytes. Due its normal phase-like mechanism, it cannot always replace reversed phase HPLC (RP-HPLC). Van der Vorst et al. questioned the sustainability of SFC when taking into account all aspects.[23] Finally to reduce the heavy demand of HPLC for toxic solvents like acetonitrile, methanol and tetrahydrofuran (THF), many studies have proposed to replace them by less toxic alternatives like ethanol,[24-27] acetone, isopropanol, propylene carbonate[24, 25]

and ethyl lactate.[28]

Almost all of the above studies concern analytical separations and relatively little attention has been paid to making preparative separations greener, probably because it is harder to make them green anyway.[12] All of the miniaturization approaches do not work, as they do not decrease the amount of solvent needed per gram of purified product. Also pHPLC precludes the use of non-volatile additives as in MLC or the use of high-boiling (and viscous) green solvents such as propylene carbonate (b.p. 242 °C) and ethyl lactate (b.p. 151 °C) and preparative high-efficiency columns with sub 2 µm particles do not exist. Thus, only replacement is an option for RP-pHPLC. However it is unclear if any less toxic alternatives could really substitute for acetonitrile, methanol and THF with respect to chromatographic selectivity and efficiency, cost and workability. The aim of this paper is to evaluate various organic solvents for their suitability in RP-pHPLC, select the best three and test them in a real-life preparative separation.

Table 1 Comparison of properties of traditional RP-HPLC organic modifiers and alternative ones

Name solvent	methanol	ethanol	acetonitrile	acetone	THF	ethyl acetate
toxicity TLV* (ppm)[29]	200	1000	40	750	200[29] 50[30]	400
safety**[31]	1.65	1.6	2.2	1.7	1.7	1.65
cost (€/L)[32]	2.82	2.41	7.20	3.44	10.56	3.54
energy to prepare (MJ/kg)[31]	41	50	88	75	271	96
elution strength [33]	3.0	3.6	3.1	3.4	4.4	***
boiling point (°C)	65	78	82	56	66	77
viscosity (cP)****	0.6	1.2	0.37	0.32	0.46	0.45
chromatogr. selectivity [34]	II	II	VI	VI	III	VI
UV (nm) transparency	> 210	> 210	> 193	> 335	> 230	> 260
odor thres-hold (ppm)[29]	100	84	170	13	2	4
PEEK compatibility	+	+	+	+	±	+
stability*****	+	+	+	±	–	±
NMR signals	+	±	+	+	±	–
solubility in water	miscible	miscible	miscible	miscible	miscible	8.3 g/100 mL

* by inhalation, TLV = Threshold Limit Value (in ppm)

** score on release potential, fire/explosion hazard and reactivity/decomposition as part of EHS evaluation of solvents; a lower score, indicates a safer solvent

*** no value could be found, our estimation is ~4.5

**** viscosity of methanol-water and ethanol-water mixtures is considerably higher than the viscosity of either pure methanol/ethanol or pure water; this is a disadvantage of methanol/ethanol

***** based on our own assessment/experience

Results and discussion

Selection of alternatives for methanol, acetonitrile and THF

Liquid chromatography (LC), and especially RP-HPLC, is the most used universal high-resolution separation technique for small organic molecules. Acetonitrile, methanol, water, and to a lesser extent THF, are the four most common solvents used for preparing RP mobile phases. In combination with 22 mm i.d. columns, a pHPLC consumes about 30 L of solvent per 24 h and produces an equal amount of waste. All three organic constituents as well as the waste are significantly toxic and potentially environmental pollutants.[35,36] "Reduce" and "replacement" are two of the most followed rules for green separations in the well-known "12 principles of green chemistry"[1] but for pHPLC only replacement is a viable option.

Unfortunately there are only few less toxic organic solvents and there are also practical considerations in pHPLC such as chromatographic selectivity (preferably different for each replacement), boiling point (< 100 °C), viscosity (< 1.5 cP), smell, cost (also reflects in part the energy requirements in its production), stability, miscibility with water, UV transparency, corrosiveness, and elution strength. Methanol is the easiest to replace as ethanol has proven itself in many analytical HPLC papers (see Introduction for references). Advantages over methanol include much lower toxicity, slightly lower costs and higher elution strength, which means that less ethanol than methanol is needed for comparable retention times. Its lower vapor pressure will lead to less evaporation and consequently to lower inhaled concentrations. Its chromatographic selectivity is the same as that of methanol, which is desired. Disadvantages include higher viscosity, harder to remove and, if not removed completely, more residual solvent signals in the NMR spectrum of the isolate. On the Pfizer list of 11

preferred solvents,[37] there are also the alcohols: n-propanol, isopropanol, n-butanol and *t*-butanol, but as these are not less toxic or cheaper than ethanol and possess much higher viscosities and boiling points, they were not considered as serious alternatives for pHPLC purposes.

The remaining solvents on the Pfizer green list are acetone, methyl ethyl ketone, ethyl acetate and isopropyl acetate. According to Snyder's classification of organic solvents (Fig. 1a), acetonitrile, acetone, methyl ethyl ketone and ethyl acetate are all in group VI, which means they have similar proton acceptor, proton donor and dipole moments.[32] However, in our experience, the experimental selectivity in RP-HPLC of solvents from the same group can still vary significantly. Of the two ketones, we have a clear preference for acetone over methyl ethyl ketone on the basis of availability as HPLC grade, cost, smell and miscibility with water. Thus we selected acetone as a replacement for acetonitrile in this study. A constraint of acetone is its UV absorbance below 335 nm. Although the working range may extend to 320 nm with short preparative UV cells, it still significantly hampers its applicability. For all non-volatile analytes an evaporative light scattering detector (ELSD), as used in this study, is an interesting alternative for pHPLC work and then acetone can be used without problems. Also a mass spectrometer, which is becoming a more and more popular detector for pHPLC, can cope with acetone.

Of the three solvents to be replaced, THF is the least desirable one to work with as it is toxic, attacks PEEK tubing, forms peroxides and is expensive. Of the two remaining solvents, we prefer ethyl acetate over isopropyl acetate for reasons of availability, cost, boiling point and water miscibility. Thus in this study ethyl acetate was used as a replacement for THF.

Although THF is in group III (Fig. 1a), actually ethyl acetate differs mainly in its dipole moment from THF.[34] With respect to the energy required to make 1 kg of solvent and CO_2 production during manufacture and incineration, ethyl acetate scores worse than ethanol and acetone but much better than THF.[38] A significant disadvantage is the poor miscibility of ethyl acetate and water, 100 mL of a saturated ethyl acetate solution contains only 9.2 mL of ethyl acetate. However in mixtures with ethanol or acetone, the solubility was not an issue in this study. The only other possible alternative for ethyl acetate is methyl acetate. The latter solvent is not on the Pfizer list[37] but does occur in other studies on green solvents, where it scores better than ethyl acetate on the basis of EHS and LCA analyses.[31] An additional advantage is its higher water solubility. Disadvantages are availability/costs, higher vapor pressure/flammability and hydrolysis to methanol instead of ethanol upon inhalation.[39] This is also reflected by the twice lower Threshold Limit Value.[29] Thus we decided to continue this study with ethanol, acetone and ethyl acetate as possible greener replacements for pHPLC. Their properties, and those of the three solvents they replace, are summarized in Table 1.

Set-up of optimization process

Nowadays reliable HPLC optimization software allows one to simulate 1 million chromatograms after recording 12 scouting chromatograms. In these 12 runs, two organic modifiers mixed with water can be tested (pure or in 1:1 ratio) as well as two temperatures and two gradient times. However the software does not allow the comparison of three different organic modifiers. We therefore resorted first to the simplex design as published by Glajch et al.[40] to find two solvent compositions, which give a fair separation of the analytes of

choice. These would then form the starting point in a further optimization step with the help of modern HPLC modelling software. This should finally lead to an optimal solvent and elution conditions, which will be tested in preparative runs.

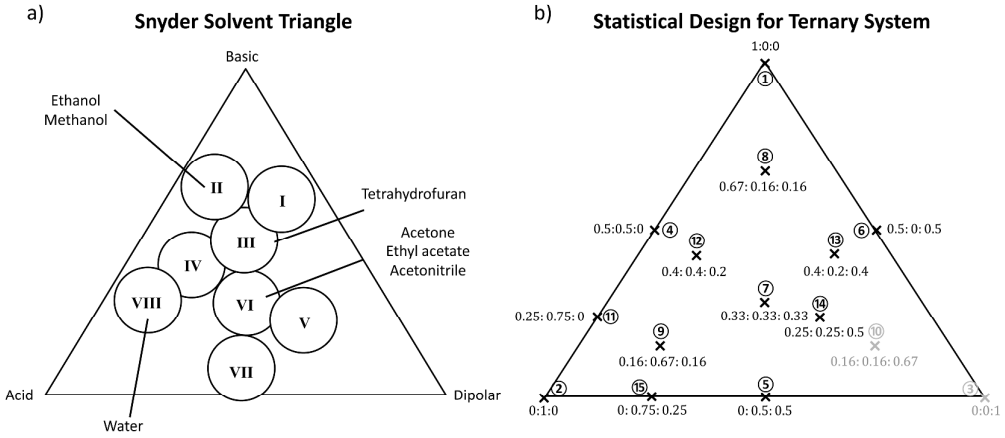

Fig. 1 a) Snyder solvent triangle; b) Simplex design for ternary system triangle. Point 1: ethanol-water (19:81); point 2: acetone-water (27:73); point 3: ethyl acetate-water (15:85). Points 3 & 10 are in grey as they cannot be prepared due to immiscibility problems.

Model analytes

A group of terpene trilactones (TTLs), i.e., ginkgolide A (GA), B (GB), C (GC), J (GJ) and bilobalide (BB) were used as the model analytes to evaluate the scope of the proposed solvents as greener mobile phases in pHPLC. There is an old but still excellent study comparing the selectivity of methanol, THF and acetonitrile for the separation of TTLs.[41] This study showed that acetonitrile-water mixtures cause co-elution of GA and GB and are best avoided. Methanol-water (30:70) and methanol-THF-water (15:5:75) gave fair separations. Later methanol-THF-water (20:10:70) was shown to give an even better separation of the five

TTLs.[42] The latter eluent was used as the benchmark solvent in this study. In combination with a 100 mm UHPLC column, the Rs_{min} was 3.13 for GC and BB while the total run time was less than 10 min. As TTLs do not significantly absorb UV light, evaporative light scattering detection (ELSD) was used, which is also perfectly compatible with the organic solvents used in this study for optimization.

Optimization by means of simplex design

In the simplex design study, 15 points on a triangle were chosen: 3 apices, 5 points on the outside and 7 points inside the triangle (Fig. 1b). Each point represents a different composition of ethanol, acetone, ethyl acetate and water. The top apex (1) is a mixture of ethanol and water, the bottom left apex (2) represents an acetone-water mixture while the most right apex is a mixture of ethyl acetate of water (Fig. 1b). For obtaining the right composition for the 3 apices (points 1-3 in Fig. 1b) several ratios of water-organic modifier were tested isocratically with the analytes and the average retention factor (k′) of the five TTLs was compared to those of the benchmark eluent methanol-THF-water (20:10:70). The results are shown in Fig. 2. The average k′ values (~4) obtained with ethanol-water (19:81; in blue) and acetone-water (27:73; in green) were close to the average benchmark k′ value (in purple). The red line presents the data for ethyl acetate. Clearly, for ethyl acetate-water (9:91) the average k′ (~8.5) is too low however it is impossible to dissolve more than 9% ethyl acetate in water. To arrive at the virtual but correct composition for the ethyl acetate/water mobile phase at apex 3, a calculation according to[43, 44] was carried out. The following Equation (1) shows the relationship between retention factor (k′) and mobile phase

199 composition (φ).

200 $$\ln k' = A\varphi^2 + B\varphi + C \quad (1)$$

201 Based on the experimentally determined relationship between k′ and φ, it is possible to
202 calculate A, B, C. If k′ ~3.8 is desired, φ should be around 0.85. Thus the virtual apex for
203 ethyl acetate-water was fixed at 15:85. This virtual composition allowed the preparation of all
204 other eluents corresponding with points on the simplex triangle (Fig. 1b) with the exception of
205 point 10. For all other points, the presence of ethanol and/or acetone sufficiently increased the
206 solubility of ethyl acetate in the total mixture.
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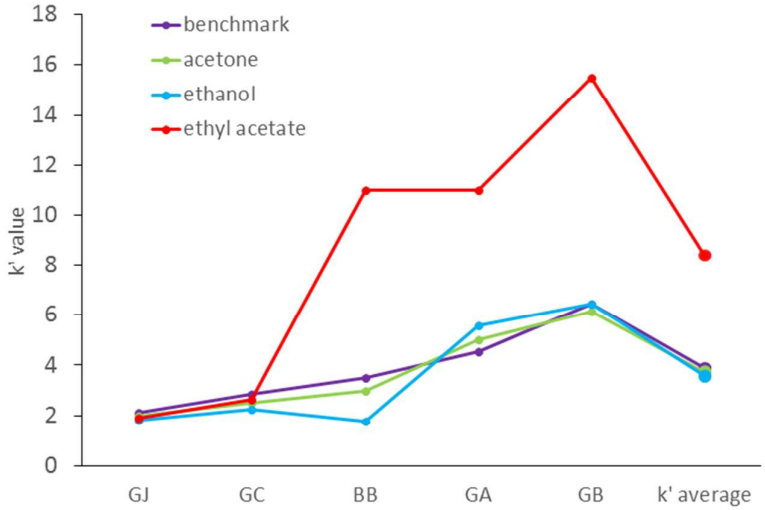

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209 **Fig. 2** Individual k′ values and average k′ value of five TTLs with different mobile phases;
210 benchmark (in purple): methanol-THF-water (20:10:70); ethanol (in blue): ethanol-water
211 (19:81); acetone (in green): acetone-water (27:73); ethyl acetate (in red): ethyl acetate-water
212 (9:91).

213 Average retention factors (k′) and minimum resolution (Rs_{min}; resolution of worst
214 separated pair of analytes) were experimentally determined in triplicate for the 13 mobile
215 phase compositions indicated in Fig. 1b. The results are shown in Fig. 3. All compositions

gave a good average k′ value (from 3.5 to 5.3), however, the minimum resolution varied from 0 to 4.4. For example, BB & GJ were not separated at Point 1 and BB & GC were not separated at Point 4. Ethanol strongly reduced the retention of BB, similar to methanol. Ethyl acetate showed the opposite effect: relative to the other TTLs, it strongly increased the retention of BB, similar to THF. Acetone did not show such effects, confirming that solvents in the same Snyder class, can exhibit a very different selectivity in RP-HPLC. Points 6, 7, 9, 13 and 15 all showed a good minimum resolution (~4) for the separation of the five TTLs. The average k′ value for each of them was also similar to the benchmark. As all of them, but point 9, are on a straight line, we assumed that the optimum solvent should be on or close to this line. Thus the solvent compositions corresponding with points 6 and 15 were selected as the starting solvents for further optimization by means of the HPLC simulation software. If an Rs_{min} of 4.4 would be considered sufficient for pHPLC, which would normally be the case, at this point it would be a valid option to skip further optimization and proceed with eluent composition 6: water-ethanol-ethyl acetate (83:9.5:7.5). This would already constitute a significant advantage over the THF and methanol-containing benchmark eluent both in toxicity and Rs_{min}. As more often than not an Rs_{min} of 4.4 will not be achieved after one round of optimization, for proof of principle we continued with the second optimization step. Apart from this, an even higher Rs_{min} is always desirable as it simply means more sample can be injected before baseline separation is lost.

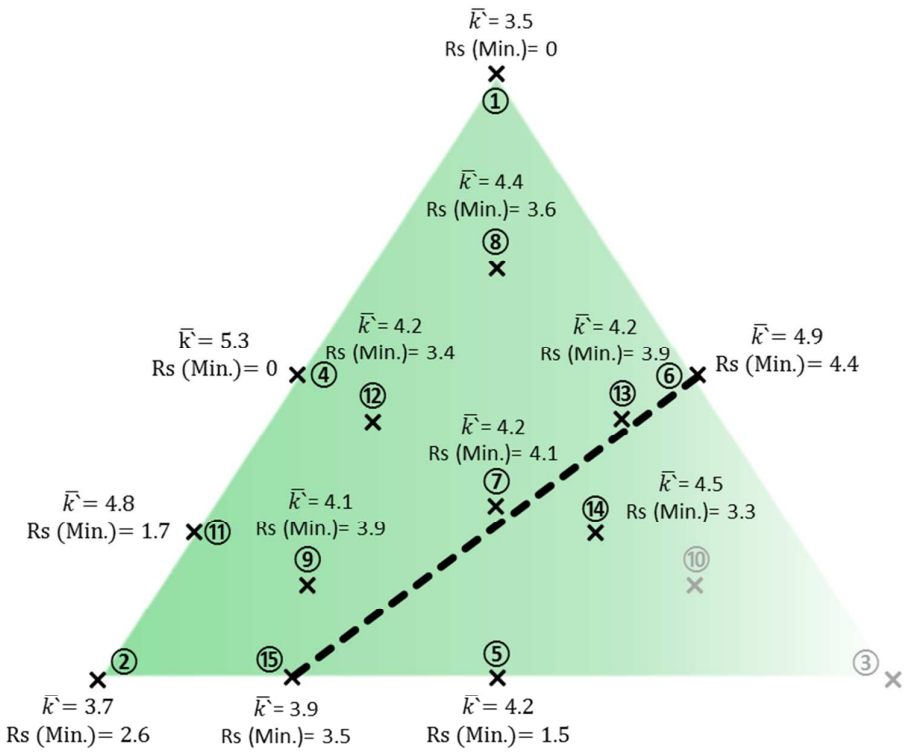

Fig. 3 Average retention factor (k′) and minimum resolution (Rs_{min}) for each mobile phase in the ternary triangle simplex system. Points 3 and 10 could not be measured because ethyl acetate and water are not miscible with each other at these ratios. Dashed line shows compositions potentially providing a high Rs_{min}.

Further optimizing mobile phase by means of HPLC simulation software

The HPLC optimization software requires slow and a fast gradient runs as input. For the 100 mm UHPLC column, 6 and 18 min are customary. This allows the software to deal with the effect of water on the chromatographic selectivity, which is something the simplex design used so far cannot take into account. Thus, the isocratic conditions corresponding with points 6 & 15 had to be converted to gradient conditions. The ratio between acetone and ethyl acetate at point 15 is 20.25:3.75 and should remain constant during the gradient, only the

248 percentage of water may vary. Similarly the ratio between ethanol and ethyl acetate at point 6
249 should remain fixed at 9.5:7.5 during all gradients. After a few scouting runs, it was
250 established that the 5 TTLs could all be eluted within 6 min by a 5% to 80% B gradient for
251 the acetone-ethyl acetate-water system. For the ethanol-ethyl acetate-water system a 5% to 70%
252 B gradient in 6 min sufficed (Table 2). To satisfy software demands and to make them more
253 uniform and reproducible, in their final form both gradients ran from 6% to 100%. This was
254 possible by adding a small percentage of water to the mixed organic phase. Finally, 12 runs
255 using 3 mobile phases for B1, B2 and 50% B2 in B1 (shown in Table 2) at 2 gradient times (6
256 min and 18 min) and 2 temperatures (25 °C and 50 °C) were carried out. Consequently 12
257 experimental chromatograms were obtained, which were imported as raw data into the
258 modelling software. In the software, B1 is mobile phase B for point 15, and B2 is mobile
259 phase B for point 6.
260
261 **Table 2** Conversion of isocratic conditions to gradient conditions and final three mobile phase
262 compositions for use with HPLC modelling software

		B1	50% B2 in B1	B2
Isocratic condition	Mobile phase	acetone-ethyl acetate-water	--	ethanol-ethyl acetate-water
	ratio	20.25:3.75:76	--	9.5:7.5:83
Gradient condition	Mobile phase A composition	water 100%		
	Mobile phase B	acetone-ethyl acetate-water	acetone-ethanol-ethyl acetate-water	ethanol-ethyl acetate-water
	composition	67.5:12.5:20	33.75:19.55: 21.7:25	39.1:30.9:30

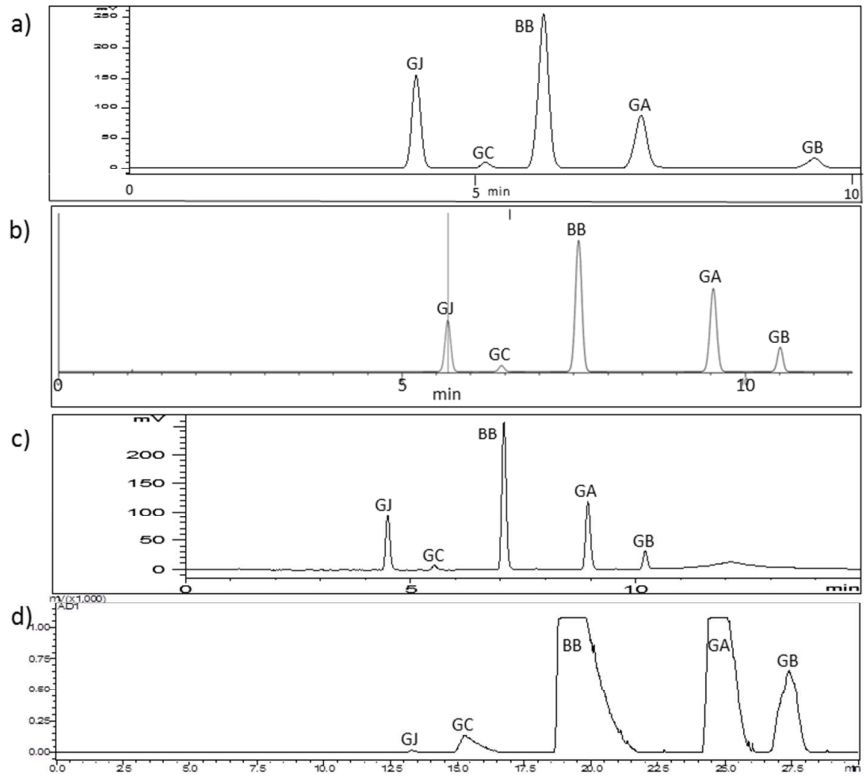

Fig. 4 a) Benchmark chromatogram of 5 TTLs with methanol-THF-water (2:1:7) as mobile phase; b) simulated chromatogram for the optimal UHPLC mobile phase; c) experimental chromatogram for the optimal UHPLC mobile phase; chromatograms a & c were obtained on a 100×2.1 mm C18 column at 0.20 mL/min; d) pHPLC chromatogram for the optimal mobile phase condition (250×22 mm C18 column, 480 mg injected).

After assigning the chromatograms, the software automatically calculates 1 million chromatograms and presents the optimal gradient. The best gradient for separation of the 5 TTLs was 22% B2 in B1 used as mobile phase B (corresponding with water-acetone-ethanol-ethyl acetate (22.2:52.65:8.6:16.55), a column temperature of 22.5 °C, and a linear gradient from 1% to 38.56% in 12.28 min). Under these conditions, the predicted minimal resolution was 4.8 (for GC and BB), which was, as expected, a bit higher than the

best Rs_{min} of 4.4 found in the simplex design study. As BB is normally the component present in the highest concentration in GBE, a higher resolution for BB with GC and GA is desirable as it would allow the injection of more sample/run. The optimum temperature of 22.5 °C means effectively room temperature, which is convenient for pHPLC as it requires some trouble and energy to pre-heat the high flow of eluent and to cool it afterwards. The software allowed for a further optimization of the gradient aiming at a higher resolution for BB, while keeping the mobile phase composition and column temperature fixed. The final stepwise UHPLC gradient (Table 3, Fig. 4b) should give a minimum resolution of 5.14 for GJ and GC with a gradient time of 10.46 min. It was selected as the best condition for the next steps of experimental verification and preparative application.

Table 3 Best gradient conditions and scale-up from UHPLC via HPLC to pHPLC

UHPLC		Analytical HPLC		Preparative HPLC	
Time (min)	B%	Time (min)	B%	Time (min)	B%
0	21	0	21	0	21
3.72	21	11.5	21	12.25	21
4.86	30	11.66	30	12.38	30
7.37	30	17.68	30	18.78	30
10.46	59	25.09	59	26.65	59
15	21	35.99	21	30	21

Experimental verification

First the best predicted condition was carried out on a real UHPLC instrument. The simulated and experimental chromatograms are compared in Fig. 4b and 4c. The predicted and experimental retention times of each TTL do not match perfectly but the deviation stays

within ± 0.6 min. Resolution and peak profile, however, are quite coherent with each other. The experimentally determined minimal resolution (5.76) was even higher than the predicted one (5.14). The worst resolution (GC and BB) obtained with the THF-methanol benchmark solvent (Fig. 4a) is only 3.13, so much lower than the minimal resolution (5.76) of the new less toxic mobile phase system. The analysis time for both conditions is around 10 min. This means the acetone-ethyl acetate-ethanol mobile phase should display a higher throughput when scaled up to pHPLC.

The UHPLC gradient was then adapted to correct for column lengths, first to analytical HPLC (4.6 mm i.d.) and then to preparative HPLC (22 mm i.d.) (Table 3). We knew from other applications that all three columns used are very comparable so no major shifts in selectivity were expected. The flow rate was also adapted to maintain the same linear flow. Preparative HPLC experiments using a Ginkgo leaf extract highly enriched in TTLs (mostly GA, GB and BB) were carried out for real-life testing of the greener eluent. The pressure during the run was approximately 150 bar. Per 30 min pHPLC run around 99 mL of acetone, 16 mL of ethanol and 31 mL of ethyl acetate were consumed besides water. After some trial runs, it proved possible to inject per run 480 mg of sample dissolved in acetone-water (1:1, v/v) while still preserving baseline separation of all five TTLs (Fig. 4d). White crystals were obtained after evaporation of the solvent *in vacuo* and recrystallization. The final yield for GA, GB, GC and BB was 98, 38, 18 and 176 mg respectively. The amount of GJ was very small. The purities of the 5 TTLs as determined by UHPLC were all above 99.5% (see SI). Quantitative NMR showed the absolute purity for GA, GB, GC and BB to be 95.3%, 96.2%, 95.7% and 95.3% respectively (see SI).

Experimental

Materials and instruments

HPLC-grade methanol, tetrahydrofuran, acetone, ethanol, acetonitrile, and ethyl acetate were purchased from Merck, Germany. The Ginkgo terpene trilactones (TTLs) ginkgolide A (GA), B (GB), C (GC), J (GJ), and bilobalide (BB) were isolated in our lab.[45] Uracil used for determining of the dead time was purchased from Sigma, the Netherlands.

The UHPLC was an Agilent 1290 Infinity, equipped with binary pumps, autosampler, thermostatted column compartment, and Sedex 90 LT-ELSD; column: Agilent Zorbax Eclipse Plus C18, 2.1×100 mm 1.8 µm; flow: 0.20 mL/min, injection volume 1.0 µL. For getting an aqueous solution saturated with ethyl acetate, ethyl acetate was gradually added to water until there was an organic layer on top of the solution. The whole solution was stirred overnight to obtain full saturation. The bottom layer was used as HPLC eluent (~9% v/v ethyl acetate in water).

The prepHPLC consisted of two LC-8A pumps, SIL-10AP auto injector, FRC-10A fraction collector, LT-ELSD and active splitter, all from Shimadzu, Japan; the column selector allowed the use of either an analytical column: Alltima C18, 250×4.6 mm 5 µm, Alltech, (the Netherlands); flow: 1.0 mL/min, injection volume 20 µL, no split; or a preparative column: Alltima C18, 250×22 mm 5 µm, Alltech; flow 20 mL/min, injection volume 4 mL, active split to ELSD, 120:1.

Drylab Software (version 4; Molnár-Institute, Berlin) was used for chromatographic solvent optimization.

Optimization of solvent strength of water – organic modifier mixtures for use in simplex design

According to Glajch et al., the first step in the solvent optimization according to the simplex design, is to determine the solvent composition at the 3 apices of the solvent triangle.[40] To this purpose various isocratic runs were carried out with mixtures of water with ethanol, acetone and ethyl acetate respectively to arrive at approximately the same solvent strength as the benchmark eluent (average k′ for the 5 TTLs ~4).

Selection of the two optimal solvent compositions by means of simplex design

The five TTLs were analyzed under isocratic conditions with the 13 solvent compositions indicated in Fig. 1b as mobile phase. Their retention factors (k′) and critical resolution (Rs_{min}) were calculated based on the obtained chromatograms (see supplementary information (SI)). The two optimal compositions corresponded with point 6 (ethanol-ethyl acetate-water=9.5:7.5:83) and point 15 (acetone-ethyl acetate-water=20.25:3.75:76) and were used for further optimization by HPLC modelling software.

HPLC eluent optimization by HPLC simulation software

To use the HPLC simulation software (DryLab, Berlin) with a 100 mm column, a number of gradient runs with two different gradient times of 6 and 18 min are required and all analytes need to elute before the end time of the short gradient (t_r < 6 min). This meant that the isocratic conditions needed to be converted to gradient conditions. This was accomplished by using water as the weak mobile phase (A) and the two best eluents from the simplex design

without water as the strong solvent (B1 and B2). The ratio of ethanol-ethyl acetate (B2) and acetone-ethyl acetate (B1) remained unchanged. In total 12 gradient separations of the 5 TTLs were carried out: 2 gradients (6 min and 18 min) × 2 temperatures (25 °C and 50 °C) × 3 different eluents (B1 (Point 15 in Fig. 1b), B2 (Point 6 in Fig. 1b) and B1-B2 (1:1)). The chromatograms were entered into the software for further optimization. One million simulated chromatograms were obtained from which the one giving the highest minimal resolution was selected.

Preparative HPLC of 5 TTLs with the optimal mobile phase

After minor adaption of the mobile phase composition and gradient, the simulated chromatogram corresponding with the optimal solvent composition was compared with the experimental chromatogram obtained by means of UHPLC. Then, the UHPLC conditions were scaled up by calculation, first to HPLC (250×4.6 mm column) and then to pHPLC (250×22 mm column).

An enriched *Ginkgo biloba* extract containing approximately 94% of TTLs (GA 27.85%; GB 11.92%; GC 5.50%; GJ 0.90%; BB 47.92%) was used as a real-life sample for evaluating the transferability of the optimal solvent. 480 mg of sample dissolved in 4 mL of acetone-water (1:1), were injected into the pHPLC. Each TTL was collected separately and the solvent was removed in vacuum by means of a rotary evaporator. The crude yield was ~85%. After recrystallization,[45] the yield of GA, GC and BB was ~75%. The purity of all 5 TTLs was assessed by means of UHPLC (area normalization) and quantitative NMR. qNMR: an accurately weighed amount of each TTL and the internal standard 1,4-dimethoxybenzene

(99.8%) was dissolved in deuterated methanol-benzene (2:1) and NMR spectra were recorded.[46]

Conclusion

After taking into account both green and practical issues of which toxicity for the user counted most, ethanol, acetone and ethyl acetate were proposed as possible replacements for methanol, acetonitrile and THF in preparative RP-HPLC. During a two-step optimization procedure it became obvious that all three alternatives exhibited significantly different selectivity, at least for our model analytes, five closely related terpene trilactones. This is desirable. The results also showed that a one-step chromatographic optimization procedure would not have led to current optimal outcome, that is, the two optimization procedures are complementary. The best separation is achieved by a gradient in which the weak solvent is water and the strong quaternary solvent is water-acetone-ethanol-ethyl acetate (22.2:52.65:8.6:16.55). This gave a minimal resolution of 5.76, which is much higher than that of the best traditional (Rs_{min} = 3.13) solvent containing THF and methanol. This shows that greener can actually mean more efficient too. An additional advantage of the green alternative is that on average its elution strength is higher meaning that less organic solvent is needed. For this reason, and because the expensive THF is not needed, in this case greener also equals cheaper. The alternative mobile phase system was successfully scaled up from 100 × 2.1 mm UHPLC to 250 × 22 mm pHPLC with a real sample while preserving baseline separation. Selectivity was not affected. All the data prove convincingly for at least this sample that alternative less toxic, cheaper solvents can work equally well if not better than traditional solvents for RP-HPLC. As the combined optimization procedure is generic, it is

405 expected that it will also work for other analyte mixtures. For other applications however the
406 proposed solvents do have their limitations: acetone is problematic if UV detection is
407 mandatory and the limited miscibility of ethyl acetate is a problem if it is the major organic
408 modifier and the analytes are non-polar. Methyl acetate might serve as a substitute for ethyl
409 acetate in some cases. The positive results might inspire others to move away from the
410 acetonitrile-methanol mindset and experiment with more benign solvents for their pHPLC
411 separations.
412

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420

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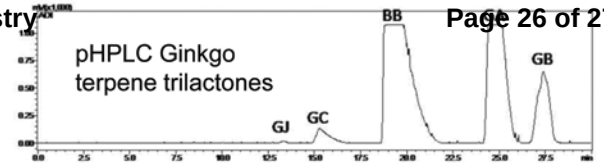
traditional RP-HPLC solvents:

alternative RP-HPLC solvents:



preparative HPLC result:
resolution ↑
toxicity ↓
cost ↓
environmental impact ↓

Novelty of the work (in 20 words):

Greener ethanol, acetone and ethyl acetate provided better chromatographic resolution in preparative RP-HPLC than the traditional methanol, acetonitrile and tetrahydrofuran.