SPECIAL GUEST EDITOR SECTION

Ultra High Pressure Liquid Chromatography for Crude Plant Extract Profiling

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Ultra high pressure liquid chromatography (UHPLC) systems operating at very high pressures and using sub-2 μm packing columns have allowed a remarkable decrease in analysis time and increase in peak capacity, sensitivity, and reproducibility compared to conventional HPLC. This technology has rapidly been widely accepted by the analytical community and is being gradually applied to various fields of plant analysis such as QC, profiling and fingerprinting, dereplication, and metabolomics. For many applications, an important improvement of the overall performances has been reported. In this review, the basic principles of UHPLC are summarized, and practical information on the type of columns used and phase chemistry available is provided. An overview of the latest applications to natural product analysis in complex mixtures is given, and the potential and limitations as well as some new trends in the development of UHPLC are discussed.

Plants are known to produce a large array of natural products (NPs) that are either essential for their life (primary metabolites) or that are not directly involved in the normal growth, development, or reproduction but are necessary for survivability, fecundity, or aesthetics (secondary metabolites). All of these compounds constitute the plant metabolome that represents an extremely complex biological matrix. Its size is not yet known but has been estimated to exceed several thousand constituents (1). Secondary plant metabolites in particular have provided the inspiration for a large number of the active ingredients in medicine. The reason for this success in drug discovery can probably be explained by their high chemical diversity, the effects of evolutionary pressure to create biologically active molecules, and/or the structural similarity of protein-binding sites across many species (2). This large chemical diversity is also directly linked to a high variability of their intrinsic physicochemical properties that render their separation, detection, and characterization challenging.

In order to characterize all these metabolites, crude plant extract profiling is essential in different domains related to plant sciences, such as fundamental biology, plant physiology, botany, agronomy, nutrition, phytotherapy, drug discovery, and systems biology. Analyzing crude extracts is a challenging task that, according to the study, requires methods providing high chromatographic resolution for detailed profiling or high throughput for rapid quantification or fingerprinting analysis. Furthermore, these methods should give online spectroscopic information for the identification of each individual metabolite for dereplication purposes.

HPLC has been recognized since the early 1980s as the most versatile technique for the efficient separation of NPs directly in crude mixtures without the need for complex sample preparation (3). HPLC has been greatly improved through the years in terms of convenience, speed, choice of column stationary phases, sensitivity, applicability to a broad variety of sample matrixes, and ability to hyphenate the chromatographic method to spectroscopic detectors (4). From the chromatography viewpoint, the development of columns with different phase chemistries (especially RP) has enabled the separation of almost any type of NPs. The recent introduction of phases stable at very high pH with small particles has considerably improved the performance of HPLC (5).

The introduction of ultra high pressure liquid chromatography (UHPLC) systems has allowed a remarkable decrease in the analysis time and an increase in peak capacity, sensitivity, and reproducibility (6). This technology, operating at very high pressures and using sub-2 μm packed columns, has rapidly been widely accepted by the analytical community at both the industrial and academic levels. The interest in UHPLC is also growing in plant sciences and, as shown in Figure 1, since 2006 the number of applications related to plant analysis has been constantly increasing while conventional HPLC methods remain relatively stable. It has to be noted, however, that the number of UHPLC applications is still much more restricted at present than those of HPLC, and the scales in Figure 1 differ greatly.

In addition, UHPLC has started to play an important role in new research fields such as metabolomics (7). This holistic approach has recently emerged with other "omics"

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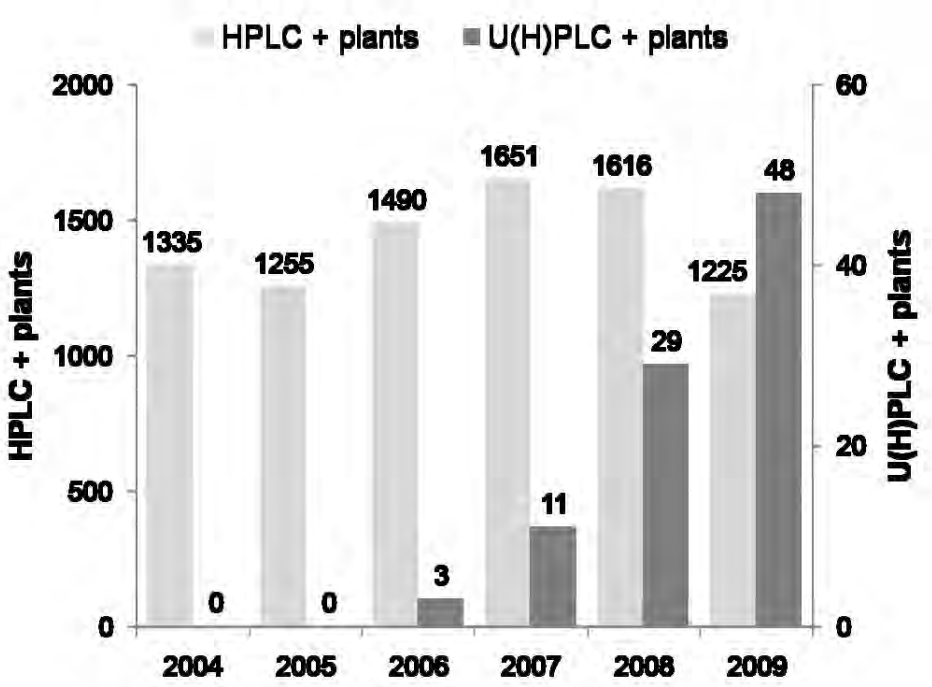

Figure 1. Number of papers by year of publication retrieved from Scifinder Scholar (Chemical Abstracts) using the keywords "HPLC" and "plants," and "UHPLC" or "UPLC" and "plants." Note that, for clarity, the scales for the two series are different. (Compiled on December 17, 2009.)

technologies in biological research (8), and can be considered as a large-scale analysis of metabolites in given organisms in different physiological states. Profiling the metabolome may provide the most functional information among the "omics" technologies used in systems biology (9). In this respect, UHPLC coupled with time-of-flight MS (UPLC/TOF-MS) represents today a key method for both metabolite fingerprinting and metabolite profiling of crude extracts. In metabolic fingerprinting, very fast separations are performed at high throughput since the intention is not to identify each observed metabolite, but to compare patterns or "fingerprints" of metabolites that change in response to disease, nutrition, toxin exposure, or environmental or genetic alterations. On the other hand, metabolic profiling focuses on the analysis of a group of metabolites related either to a specific metabolic pathway or a class of compounds. In most cases, metabolic profiling is hypothesis-driven rather than hypothesis-generating (10).

UHPLC is also used more and more for dereplication purposes in drug discovery programs in conjunction with both photodiode array (PDA) and MS detection. Dereplication is the process of differentiating those natural product extracts that contain nuisance compounds, or known secondary metabolites, from those that contain novel compounds of interest (11). Here, the high-resolution capacity of UHPLC is required for the deconvolution of closely related metabolites (such as isomers) for obtaining high-quality online spectra without interferences for database searching or spectral interpretation. Such a process represents an important step in drug discovery programs, because the early structural determination of known NPs avoids their time-consuming isolation, and enables the optimization of bioactive-guided isolation procedures (12).

Finally, UHPLC has also now conquered domains related to the QC of plants, especially for the standardization and safety assessment of medicinal plants, phytomedicines, or dietary supplements. In this respect, standard HPLC procedures are gradually being replaced by high-throughput, targeted UHPLC quantitative methods (13). Untargeted QC methods based on the principle of fingerprinting are also more frequently used to assess phytoequivalence (13, 14).

In order to assess the potential of this new technology for crude extract profiling, this review will briefly summarize the main characteristics of the chromatographic method and discuss different applications that have recently been reported in various fields of plant analysis. Practical aspects related to the type of phases and the UHPLC instrumentation available, as well as the transfer of methods from HPLC to UHPLC, will be discussed.

UHPLC

Brief Summary of UHPLC Technology

It is well known that reduction of the packing particle size in LC has a similar effect on the separation as a decrease in the column id in GC. Indeed, it is possible in LC, with smaller particles, to attain a higher plate count (i.e., efficiency inversely proportional to the particle diameter, dp) and to decrease greatly the analysis time (i.e., optimal flow rate

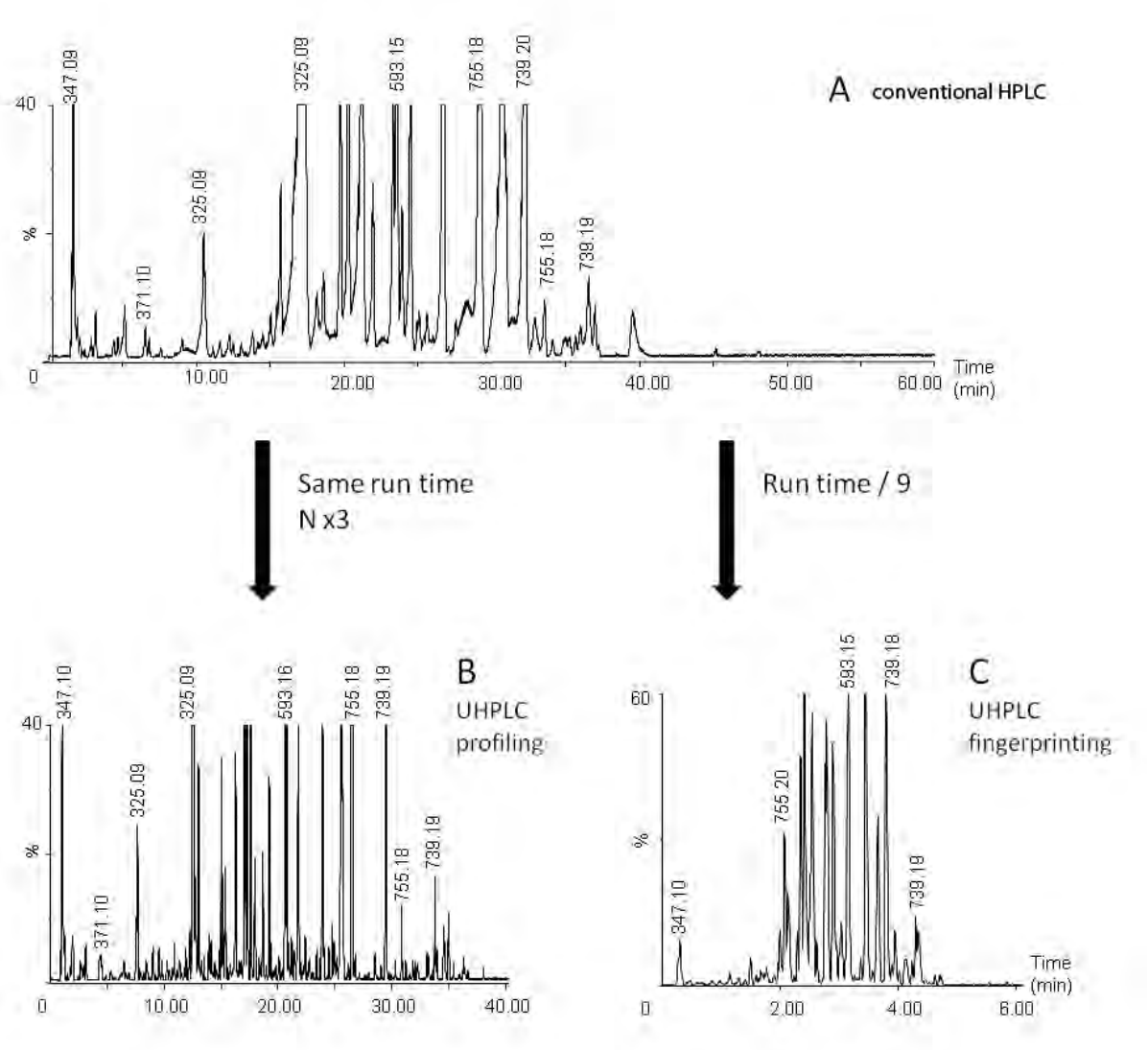

Figure 2. Comparison of chromatograms of a standardized *G. biloba* extract with transfer of method. (A) Classical HPLC analysis carried out on a 150 × 4.6 mm id, 5 μm column with gradient of 5–40% ACN (0.1% formic acid) in 60 min at 1 mL/min. (B) HPLC method transferred on a 150 × 2.1 mm id, 1.7 μm UHPLC column, with the same run time, flow reduced to 0.35 mL/min. In this case, the resolution is notably increased. (C) Geometric transfer of method calculated by HPLC modeling software on a 50 × 2.1 mm id, 1.7 μm UHPLC column with the same phase chemistry; the gradient time was reduced to 6.76 min (i.e., 9-fold reduction), and the flow rate to 0.6 mL/min. Note that the performance should be the same, but it is slightly lower because of a relatively larger dead volume due to the electrospray ionization (ESI) probe. Detection: ESI-TOF-MS, *m/z* range 100–1000 in the negative mode.

inversely proportional to dp; 15). Thus, since the early times of LC, there has been a continuous tendency toward particle size diminution to enhance chromatographic performance, from 100 to 200 μm in the 1950s to 10 μm in the 1970s, 5 μm in the 1980s, 3 to 3.5 μm in the 1990s, and finally down to sub-2 μm at the beginning of the 21st century (6). The only limitation in this strategy, except for the difficulty in homogeneously packing such small porous particles, is the generated backpressure. The latter is inversely proportional to dp^2 according to Darcy's law. In addition, since the flow rate also should be increased inversely to the particle size, the backpressure is roughly inversely proportional to dp^3 in optimal flow rate conditions (16). For this reason, when very small particles (i.e., sub-2 μm) are used, dedicated instrumentation that withstands pressures higher than 400 bar is required. The approach, which consists of using columns packed with sub-2 μm in conjunction with pressures beyond 400 bar, available since 2004, is known as UHPLC (17).

By adequately selecting the column length in UHPLC, it is possible, from a theoretical point of view, to increase the throughput by a factor of 9 compared to conventional HPLC. For example, if the original separation has been carried out on a 150 mm, 5 μm column, a 50 mm, 1.7 μm stationary phase should be selected in UHPLC to attain equivalent

Table 1. Summary of UHPLC systems, with the launch year, pressure tolerance, maximal flow rate, oven temperature, and UV acquisition rate[a]

Launch year	Provider	Name of the system	Pumping system	Pressure tolerance, bar	Maximal flow rate, mL/min (corresponding pressure, bar)	Maximal oven temperature, °C	UV acquisition rate, Hz
2004	Waters	Acquity UPLC	High pressure	1000	2 (600)	90	80
2006	Agilent	Series 1200	High pressure	600	5 (600)	100	80
2006	Jasco[b]	Xtreme-LC	High pressure	1000	3.5 (1000)	65	100
2006	Rheos[c]	Allegro UHPLC	Low pressure	1000	1 (1000)	95	20
2006	Thermo[d]	Accela 1000 system	Low pressure	1000	2 (1000)	95	80
2007	Shimadzu[e]	Prominence UFLCxr	High pressure	660	5 (440)	85	50
2007	VWR[f]	Lachrom Ultra	High pressure	600	5 (600)	85	100
2008	Dionex[g]	Ultimate 3000	High/low pressure	800	5 (800)	110	100
2008	Knauer	PLATINblue	High/low pressure	1000	5 (800)	140	200
2008	PerkinElmer[h]	Flexar FX-10 (eq. Series 275Hres)	High pressure	690	3 (600)	90	100
2009	Agilent	Series 1290 Infinity	High pressure	1200	5 (800)	100	160
2009	PerkinElmer	Flexar FX-15	High pressure	1240	5 (1240)	90	100
2009	Thermo	Accela 600 system	Low pressure	600	5 (600)	95	80
2010	Waters	Acuity UPLC H-class	Low pressure	1000	2 (600)	90	80

[a] The information presented was gathered from advertising and from providers' Websites in February 2010.
[b] Tokyo, Japan.
[c] Flux Instruments, Reinach BL, Switzerland.
[d] Thermo Fisher Scientific Inc., Waltham, MA.
[e] Kyoto, Japan.
[f] West Chester, PA.
[g] Sunnyvale, CA.
[h] Waltham, MA.

performance, but with a 9-fold reduction of analysis time because of the 3-fold shorter column and 3-times higher linear velocity (18). Such ultrafast separations have been experimentally demonstrated, and analysis times in the range of 1–5 min can be expected (19–21). On the other hand, by keeping strictly identical column lengths in both HPLC and UHPLC, it is hypothetically possible to increase the plate count by a factor of 3 between columns packed with 5 and 1.7 μm particles, and to reach up to 40 000 theoretical plates with a 150 mm, 1.7 μm packing. However, it becomes difficult to work in optimal flow rate conditions because of the substantial backpressure generated by long columns packed with sub-2 μm particles. Some separations involving 150 mm or even longer UHPLC columns have been reported in the literature and show very elevated efficiency (22, 23).

One of the main advantages of UHPLC over the other strategies proposed to increase throughput or resolving power in LC (e.g., monolith and fused-core columns and high-temperature LC) is the possibility to easily transfer existing methods from HPLC. Because UHPLC consists essentially of a change of column dimensions (i.e., length, dp, and id), the equations for geometrical changes usually used for scale-up between analytical and preparative modes can also be used to find the mobile phase flow rate, injection volume, and gradient profile to be used in UHPLC (24–26). This task can be automated by using various calculations freely available on supplier or academic Websites. By applying such rules, it is possible to maintain identical performance and selectivities between HPLC and UHPLC, provided that the selectivity of the support is identical between the initial and final approach.

An example of transfer that can be obtained from HPLC to UHPLC conditions for profiling the standardized extract of a very widely used phytomedicine, *Ginkgo biloba*, is shown in Figure 2. As expected from theory, on such a complex biological matrix, a 9-fold reduction in analysis time can be obtained by transferring the 60 min HPLC gradient (on a 150 × 4.6 mm id, 5 μm column) to a short UHPLC gradient (on a 50 × 2.1 mm id, 1.7 μm UHPLC column), while the use of UHPLC column (150 × 2.1 mm id, 1.7 μm) with the same gradient time provides a notable increase in resolution.

Table 2. Summary of providers for columns packed with sub-2 μm particles, with the number of available chemistries, the main types of chemistry, pressure tolerance, and particle size[a]

Provider	Name	Number of chemistries	Main types of chemistry	Pressure tolerance, bar	Particle size, μm
			500 bar < ΔP_{max} <800 bar		
Agilent	RRHT	8	C18, C8, CN	600	1.8
Grace-Davison[b]	Vision-HT	6	C18, HILIC, silica	800	1.5
Sepax Technologies[c]	Sepax UHPLC	11	C18, C8, C4, phenyl, amino, CN, SCX,[d] SAX,[e] HILIC	600	1.8
VWR	LaChromUltra	1	C18	600	2
YMC[f]	UltraHT	2	C18	500	2
Zirchrom Separations[g]	Zirchrom-PBD and -Phase	2	Zirconia-based material	700	2
			ΔP_{max} ≥1000 bar		
Agilent	RRHD	2	C18	1200	1.8
ES Industries[h]	Epic	7	C18, PFP, HILIC, diol, silica + 3 phases SFC[i]	1000	1.8
Interchim[j]	Strategy	2	C18, HILIC	1000	1.7
Knauer	BlueOrchid	7	C18, C8, PFP, phenyl, CN, silica	1000	1.8
Macherey-Nagel[k]	Nucleodur	5	C18, C8	1000	1.8
Restek[l]	PinnacleDB and Ultra II	12	C18, C8, PFP, biphenyl, CN, silica	1000	1.9
Thermo	Hypersil GOLD	11	C18, C8, C4, PFP, CN, phenyl, amino, AX,[m] SAX, silica	1000	1.9
Waters	Acquity BEH	6	C18, C8, phenyl, HILIC, amide	1000	1.7
Waters	Acquity HSS	3	C18	1000	1.8

[a] The information presented here was gathered from advertising and from providers' Websites in February 2010.
[b] Deerfield, IL.
[c] Newark, DE.
[d] SCX = Strong cation exchange.
[e] SAX = Strong anion exchange.
[f] Kyoto, Japan.
[g] Anoka, MN.
[h] West Berlin, NJ.
[i] SFC = Supercritical fluid chromatography.
[j] Montluçon, France.
[k] Düren, Germany.
[l] Bellefonte, PA.
[m] AX = Weak anion exchange.

Chromatographic Systems and Stationary Phases Available for UHPLC

Today, there is a wide choice of instruments that withstand pressures above 400 bar and accommodate columns packed with sub-2 μm from various suppliers, as reported in Tables 1 and 2.

Regarding the selection of a UHPLC system (Table 1), the cost is certainly a decisive consideration, but it is also important to analyze in detail the specifications of all available instruments on the market, as none of them are equivalent. The most important feature is certainly the maximal available pressure (ΔP_{max}) and corresponding flow rate, which mostly defines the price of a UHPLC system. For the commercial apparatus, the ΔP_{max} varies between 600 and 1200 bar. It has been demonstrated that for fast or ultrafast separations of simple mixtures, the use of small particles was obvious, but there was no need to work with very elevated pressures (27, 28). For such high-throughput experiments, the UHPLC instruments with pressure limits around 600 bar provide a suitable solution at a reasonable price. On the other hand, for complex crude plant extracts necessitating high-resolution separation, long columns packed with sub-2 μm particles have to be used and, thus, a system with maximal pressure of 1000–1200 bar is mandatory to work at acceptable flow rates (27, 28). In addition to the pressure capability of the apparatus, it is also important that the instrument is adapted to operate in fast and ultrafast modes with reduced column

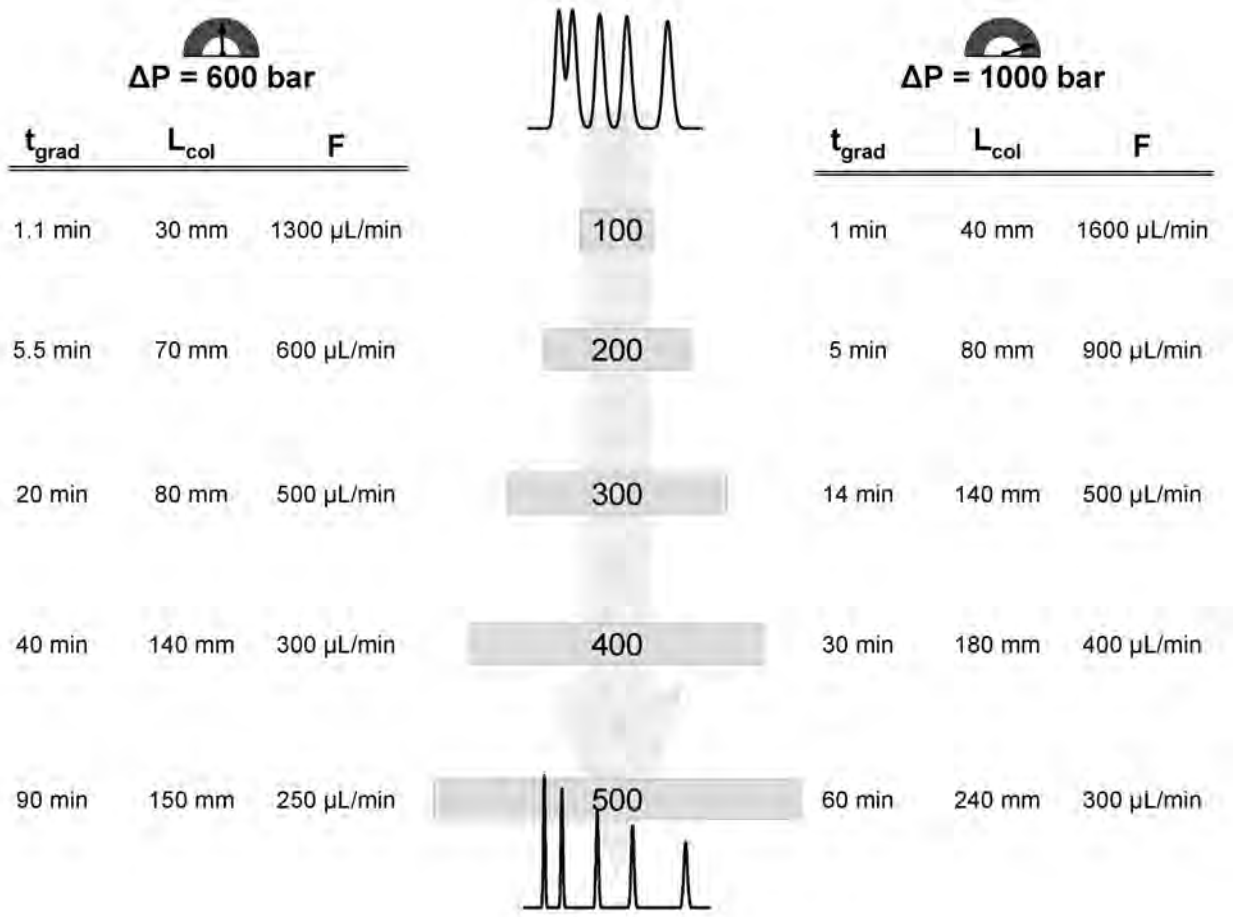

Figure 3. Comparison of the performance that can be achieved with a UHPLC system having maximal pressures of 600 and 1000 bar. Gradient times (t) and corresponding column length (L) and mobile phase flow rate (F) were all calculated for rutin (MW = 610 g/mol) for a 5–95% ACN gradient at 30°C.

volumes. For this purpose, available devices generally present low-system dead volume (i.e., reduced injection volume, UV cell volume, and tubing length and id); high acquisition rate (up to 200 Hz); and small-gradient delay volume (less than 100 µL for a few instruments). A comparative study made by a pharmaceutical company of various UHPLC systems can be found elsewhere (29).

Another important aspect when selecting a UHPLC setup is the selection of stationary phases that provide sufficient selectivity as well as acceptable performance and lifetime. An exhaustive list of available stationary phases packed with sub-2 µm particles is given in Table 2. As shown, the number of columns is quite large, with around 80 supports and more than 10 phase chemistries available from more than 10 different providers, demonstrating the opportunity to transfer almost all existing methods from HPLC to UHPLC. All of these stationary phases are not equivalent in terms of pressure tolerance (from 600 to 1200 bar), particle size (from 1.5 to 2 µm), and pH and temperature range. Some performance comparisons among the different phases can be found in the literature (30, 31), and data for column lifetime were also published (32). In our laboratory, we observed that lifetimes of UHPLC and regular HPLC columns were comparable, and 500 to 2000 injections can be performed on a single column. However, this result can strongly depend on the nature of the analyzed samples, the supplier, and the pressure range used. Regarding the problematic nature of NPs, the variety of phase chemistry can resolve almost all analytical issues (Table 2): bonded C8 and C18 for plant extracts of average polarity; bonded C4 and cyano (CN) for the most apolar fractions; diol, amino, silica, and hydrophilic interaction LC (HILIC) for the most polar fractions; and biphenyl, pentafluorophenyl, or zirconia for alternative selectivity.

Analytical Conditions for Optimal Performance in Gradient UHPLC

When dealing with extracts containing NPs, the gradient mode should be selected because of the complexity and wide polarity range of the sample. The gradient performance index is the peak capacity (P), which represents the number of peaks that can be separated with a resolution of 1 during the gradient time (33). Figure 3 summarizes the best analytical conditions in terms of gradient duration, column length, and mobile phase flow rate to reach peak capacities between 100 and 500

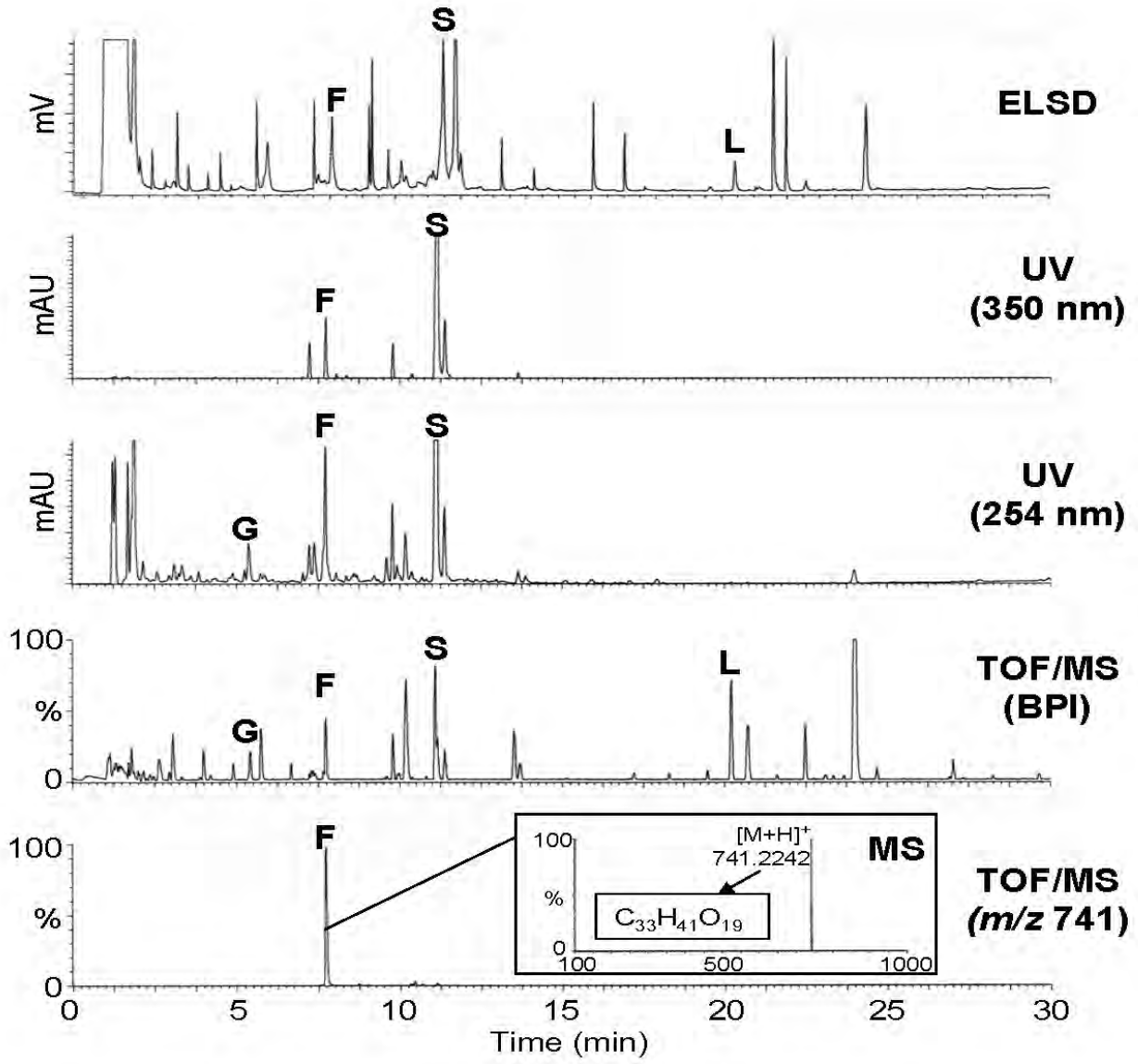

Figure 4. Comparison of different detection techniques used to monitor the UHPLC separation of the crude leaf extract of *A. thaliana*. UHPLC conditions: column Waters Acquity (BEH C18: 150 × 2.1 mm id, 1.7 μm), gradient 5 to 98% ACN (0.1% formic acid) in 45 min, temperature 35°C, flow rate of 300 μL/min. ESI-TOF-MS detection in the *m/z* range 100–1000 with a scan time of 0.25 s. UV detection at 254 and 350 nm. ELSD detection: SEDERE Sedex 85 (Alfortville Cedex, France), P_{neb} = 3 bar (P_{neb} = nebulization pressure), T_{evap} = 50°C (T_{evap} = evaporation temperature), gain 8. Compound F is a flavonol glycoside, G a glucosinolate, S a synapoyl derivative, and L a galactolipid. Inset: TOF-MS spectrum of F. Adapted from ref. 5 with permission of Thieme (New York, NY).

with a UHPLC apparatus possessing a maximal pressure drop of 600 and 1000 bar, respectively. All the calculations were performed using a methodology recently described elsewhere (34), and rutin was selected as a model compound because it is present in numerous plant extracts and possesses an average MW (i.e., 610 g/mol; 35).

It is important to remember that in the UHPLC gradient mode, the longest column does not necessarily provide the highest peak capacity, and column length (L_{col}) should be selected according to the gradient time (34). Another important factor is that UHPLC gradient experiments should be ideally performed with the highest possible flow rate in order to maximize performance (34, 36).

Figure 3 shows that the gradient time required to attain a peak capacity lower than 200 is similar for ΔP_{max} of 600 and 1000 bar (i.e., a difference of only 10%). As discussed previously, there is not much interest in using an instrument compatible with very high pressure for such high-throughput experiments. On the other hand, the extension of maximal pressure capabilities becomes

Table 3. UHPLC analyses of plant and crude extracts classified according to the type of studies performed

Column chemistry	Column size, length, mm × mm id, mm	Analysis time, min[a]	Detection[b]	Quantitative [yes (y)/no (n)]	Matrix/plant/extract	Analytes	Year	Ref.
				QC: targeted analysis				
Acquity BEH C18	100 × 2.1	5/20	MS/MS	y	*Trollius ledibouri* Reichb. and 4 commercial samples	Flavonoids and others	2006	45
Acquity BEH C18	100 × 2.1	1	PDA	y	*Hordeum vulgare* and *Pachyrhizus erosus*	*N*-acylhomoserine lactones	2007	46
Acquity BEH C18	50 × 2.1	12	PDA	y	*P. notoginseng*	Saponins	2007	47
Acquity BEH C18	50 × 2.1	4.4	PDA	y	*Taxus* spp.	Taxanes	2008	48
Acquity BEH C18	100 × 2.1	15	PDA-MS	y	*Hoodia* spp.	Various glycosides	2008	49
Acquity BEH C18	50 × 2.1	15	PDA	y	*Epimedium* spp.	Flavonoids	2008	50
Acquity BEH C18	50 × 2.1	5	PDA-MS/MS	y	*Coptis chinensis* Franch	Alkaloids	2008	51
Acquity BEH C18	100 × 2.1	20/8	PDA-MS/MS	y	*Isatis indigatica*	Flavonoids	2008	52
Acquity BEH C18	50 × 2.1	10	PDA	y	*Salvia miltiorrhiza*	Diterpenoids	2008	53
Acquity BEH C18	100 × 2.1	13	TOF-MS	y	*Eleutherococcus senticosus*	Saponins	2008	54
Acquity BEH C18	50 × 2.1	3	PDA	y	*Rheum* spp.	Anthraquinone derivatives	2008	55
ZorBax SB C18	50 × 4.6	18	TOF-MS	y	Compound Danshen preparations	Various	2008	56
Acquity BEH C18	100 × 2.1	7	ELSD-MS	y	*Actaea racemosa* L.	Formononetin and triterpenoid glycosides	2009	38
Acquity BEH Shield RP18	50 × 2.1	7	MS	y	*G. biloba*	Terpenelactones	2009	57
Acquity BEH C18	50 × 2.1	21	MS	y	*P. notoginseng*	Saponins	2009	58
Acquity BEH C18	100 × 2.1	3.5	QTOF-MS	n	*Rosaceae* spp.	Phenolic compounds	2009	59
Zorbax Eclipse Plus C8	50 × 4.6	3.5	PDA	y	*Coptis* spp.	Alkaloids	2009	60
Acquity BEH C18	100 × 2.1	13	QTOF-MS	y	*Salvia* spp.	Diterpenoids	2009	61
Acquity BEH C18	100 × 2.1	20	PDA	y	*Camellia sinensis* (L.) Kuntze	Phenolic compounds	2010	62
				QC: untargeted analysis				
Acquity BEH C18	100 × 2.1	5/9.5	MS/MS	n	24 commercialized preparations of Chinese herb	Aristolochic acids	2007	63
Acquity BEH C18	100 × 2.1	16	PDA	n	*S. Miltiorrhizae* Radix	Various	2007	64
Acquity BEH C18	150 × 2.1	10	TOF-MS	n	*C. sinensis* L.	Epicatechins	2008	65
Acquity BEH C18	100 × 2.1	10	QTOF-MS	n	*C. sinensis* L.	Polyphenols and others	2009	66

Table 3. (*continued*)

Column chemistry	Column size, length, mm x mm id	Analysis time, min[a]	Detection[b]	Quantitative [yes (y)/no (n)]	Matrix/plant/extract	Analytes	Year	Ref.
				Metabolomics				
Acquity BEH C18	100 × 2.1	11	TOF-MS	n	*P. notoginseng*	Ginsenosides and others	2007	67
Acquity BEH C18	150 × 2.1	111	TOF-MS	n	*A. thaliana*	Oxylipins	2007	68
Acquity BEH C18	50 × 2.1	7.5	PDA	y	*Trifolium* spp.	Phenolic compounds	2007	69
Acquity BEH C18	50 × 2.1	21	QTOF-MS	n	*P. notoginseng*	Saponins	2008	70
Acquity BEH C18	100 × 2.1	19	MS	n	*Taxus* spp.	Taxanes	2008	71
Acquity BEH C18	50 × 1.0 and 150 × 2.1	10 /120	TOF-MS	y	*A. thaliana* and *P. canadensis* Moench cv Robusta	Oxylipins	2008	72
Acquity BEH C18	150 × 2.1	97	TOF-MS	n	*A. thaliana*	Oxylipins	2008	73
Acquity BEH C18	150 × 2.1	107	TOF-MS	n	*A. thaliana*	Oxylipins	2008	74
Acquity BEH C18	50 × 1.0 and 150 × 2.1	10/119/325	TOF-MS	n	*A. thaliana*	Oxylipins	2008	28
Acquity BEH C18	100 × 2.1	23	QTOF-MS	n	Strawberry (*Fragaria x ananassa* cv. Jonsok)	Polyphenols	2008	75
Acquity BEH C18	100 × 2.1	20	QTOF-MS	n	*Panax* spp.	Saponins	2008	76
Acquity BEH C18	100 × 2.1	20	QTOF-MS	n	*Panax* spp.	Saponins	2008	77
Acquity BEH C18	50 × 2.1	17	PDA-QTOF-MS	n	Wen-Pi-Tang	Phenolic compounds, alkaloids, saponins, and flavonoid glycodide	2008	78
Acquity BEH C18	150 × 2.1	Various	TOF-MS	n	*A. thaliana*	Oxylipins	2009	41
Acquity BEH C18	100 × 2.1	23	QTOF-MS	n	Strawberry *(Fragaria x ananassa* cv. Jonsok)	Phytoalexins	2009	79
Acquity HSS T3	100 × 2.1	15	TOF-MS	n	*Brassica oleracea*	Coumaroylquinic acids	2009	80
Acquity BEH C18	100 × 2.1	15	MS/MS	y	*Oryza sativa* L.	Various plant hormones	2009	81
Unknown	Unknown	Unknown	MS/MS	n	*A. thaliana*	Glucosinolates	2009	82
Unknown	Unknown	2.1	MS/MS	y	14 different plants	Various	2009	83
Acquity BEH C18	50 × 1.0	7	TOF-MS	n	*A. thaliana*	Oxylipins	2010	84
				Other analysis				
Acquity BEH C18	100 × 2.1	20/8	PDA	n	Fructus aurantii	O-diglycosyl flavanones	2006	85
Acquity BEH C18	50 × 2.1	4.5	PDA	y	*Aesculus hippocastanum*	Flavonoids	2007	86

Table 3. (*continued*)

Column chemistry	Column size, length, mm x mm id	Analysis time, min[a]	Detection[b]	Quantitative [yes (y)/no (n)]	Matrix/plant/extract	Analytes	Year	Ref.
Acquity HSS T3	100 × 2.1	12	QTOF-MS	n	*Lupinus angustifolius* L.	Malonylated glycosides of flavonoids	2008	87
Acquity BEH C18	50 × 2.1	8	MS/MS	y	*A. thaliana* and *P. canadensis* Moench cv Robusta	Cytokinins	2008	88
ZorBax SB C18	50 × 4.6	19	TOF-MS	n	Danggui Buxue Tang preparation	Phenolic acids, saponins, isoflavonoids	2008	89
Acquity BEH C18	100 × 2.1	12	QTOF-MS	n	*Garcinia yunnanenis*	Benzophenones and xanthones	2008	90
ZorBax SB C18	50 × 3.0	30	PDA-TOF-MS	n	*Stellera chamaejasme*	Various	2008	91
Acquity BEH C18	100 × 2.1	23	QTOF-MS	n	*Fragaria* x *ananassa* cv. Jonsok	Polyphenols	2009	92
Acquity BEH Shield RP18	100 × 2.1	30	PDA	n	*Rhizoma coptidis*	Various	2009	93
Acquity BEH C18	100 × 2.1	3/8	MS/MS	y	*Nicotiana tabacum* cv. Xanthi nc	Jasmonic and salicylic acids and various	2009	94
Acquity BEH C18	50 × 2.1	17	MS/MS	y	*Digitalis purpurea* and *Nicotiana tabacum* and *Inula helenium*	Steroid hormones	2009	95
Acquity BEH C18	150 × 2.1	16	TOF-MS	n	*Gentianaceae* spp.	Flavonoids and others	2009	96
Acquity BEH C18	100 × 2.1	10	QTOF-MS	n	*Corydalis yanhusuo* WT Wang	Alkaloids	2009	97
Shim-pack XR-ODS	50 × 2.0	20	PDA	y	Standards	Taxanes	2009	98
Acquity BEH C18	150 × 2.1	8	PDA-MS/MS	y	*N. tabacum* L.	Absicisic acid and metabolites	2009	99

[a] Gradient time without wash and equilibration time. The presence of two (or three) gradient time values indicates a two- (or three-) step method, or different conditions for qualitative and quantitative analysis.

[b] MS/MS = Tandem MS/MS experiments performed on a triple-quadrupole MS analyzer.

mandatory to generate peak capacity between 300 and 500, corresponding to a high-resolution experiment. The gradient time is around 1.5-fold longer for a system with ΔP_{max} of 600 bar compared to 1000 bar. For the sake of comparison, a 50 cm column packed with 5 μm particles and a gradient time of 90 min would be necessary to attain a peak capacity of 300 on a conventional HPLC system (i.e., ΔP_{max} of 400 bar), while more than 300 min and a 1 m column length would be required to attain a capacity of 500.

Figure 3 also summarizes the column length and mobile phase flow rate required to attain, as quickly as possible, a given resolving power. As shown, column length should be increased simultaneously with the required peak capacity, while mobile phase flow rate should be set to the highest possible value that produces the maximal backpressure supported by the UHPLC instrument. Even for long analysis times (i.e., >60 min in UHPLC), there is no need to increase UHPLC column length beyond 250 mm.

Detection Modes Used with UHPLC

As expected, the UHPLC strategy can be coupled with any detector commonly used in conventional LC. Two main types of detectors can be defined: simple detectors able to record chromatographic traces [UV-Vis and evaporative light-scattering detector (ELSD)] and detectors that generate multidimensional data (i.e., chromatographic and spectroscopic) for online identification and dereplication purposes [UV-diode array detector (DAD) and MS; 5]. Because of the narrow peaks produced by UHPLC (down to only 1 s in ultrafast conditions), it is important to have detectors with sufficiently high acquisition rates to adequately define chromatographic peaks. In addition, because the column volume has been greatly reduced in UHPLC versus HPLC, the detector should contribute in a limited proportion to the extra-column peak broadening.

Among all HPLC detectors, the simplest and most widely used is the UV detector. It is quite easy to optimize UV-Vis and UV-DAD detectors to meet the requirements of UHPLC in terms of sampling rate. As shown in Table 1, acquisition rates can be extended up to 200 Hz for UV-Vis at fixed wavelength (i.e., PLATINblue; Knauer, Berlin, Germany) and up to 160 Hz for UV-DAD with multiple-wavelength and full-spectral detection (i.e., Agilent 1290 Infinity; Waldbronn, Germany). On the other hand, the UV cell volume should be reduced to avoid peak dispersion in UHPLC, but the path length of the light passing through the UV cell should remain sufficient, because the absorbance is directly proportional to path length according to the Beer-Lambert law. Generally, the UV cell in conventional HPLC has a volume between 10 and 25 μL for a path length of 10 mm, whereas it was reduced down to 0.5–3 μL in UHPLC for a path length of 3–10 mm, depending on the provider. The Acquity UPLC (Waters Corp., Milford, MA) and Agilent 1290 Infinity systems use an alternative UV cell technology to attain high light transmission in conjunction with long path length and small cell volume (e.g., only 0.5 μL volume and 10 mm path length for the Waters Acquity UPLC system).

The ELSD is another attractive detector for UHPLC profiling of crude plant extracts, as it is quasi-universal and able to detect chromophore-lacking compounds. Because the detection is based on the measurement of light scattering (using a photomultiplier or a photodiode) produced by the nonvolatile residual particles after evaporation of the mobile phase, the sampling rate is generally not critical (equal to at least 50 Hz in any of the commercial devices) and is sufficient even for ultrafast experiments. As recently reported (37, 38), the coupling of UHPLC with an ELSD is possible, but the latter remains a non-negligible source of additional dispersion that increases with higher mobile phase flow rates. Thus, UHPLC-ELSD for ultrafast separations has to be considered with caution, while it is more straightforward in the case of high-resolution separations. Figure 4 shows the complementarity of UV and ELS detection for UHPLC experiments. The ELSD response provides more peaks than those detected in UV at 254 and 350 nm, especially for the detection of nonpolar compounds (mainly lipids in this example; 5).

The coupling of UHPLC with MS provides chromatographic and spectroscopic information (i.e., MW, molecular formula, and diagnostic fragments). It appears to be the best approach in terms of sensitivity, selectivity, and peak assignment for the determination of analytes at low concentrations in complex matrixes such as plant extracts (39, 40). Two main types of MS analyzers were used in the field of crude plant extract profiling, namely, quadrupole and TOF. The former operating in the selected-ion monitoring (SIM) or selected-reaction monitoring (SRM) mode was preferentially selected for targeted analysis (e.g., QC), while the latter was particularly useful for nontargeted analysis (e.g., metabolomics). Regarding quadrupole-based analyzers, the sampling rate can be an issue, and modern instruments possessing improved acquisition rates should be selected for hyphenation with UHPLC. With this new analyzer generation, dwell times have been reduced to 5 ms (e.g., Waters) and even 1 ms (e.g., Agilent) in SIM and SRM modes (40). TOF instruments are also well adapted to record and store data over a broad mass range without compromising sensitivity. With the latest generation of TOF-MS instruments, high mass resolution (e.g., higher than 10 000 full width at half maximum) can be attained at speeds of 20 full spectra/s [e.g., Bruker maXis (Billerica, MA) and Waters LCT-Premier XE] and up to 40 spectra/s (e.g., Agilent 6200 Series). Aside from the acquisition rate, it has been demonstrated that MS instruments represent a non-negligible source of extra-column band broadening in UHPLC compared to the UV detector (41). In the gradient mode, it was observed that the peak capacity was reduced by 15–30% with TOF-MS compared to UV detection because of the important ionization chamber volume, transfer capillary volume, and critical electronic signal treatment (41). In addition, even though fast polarity switching ± (i.e., 20 ms) and/or fast electrospray ionization/atmospheric pressure chemical ionization (ESI/APCI) mode switching (i.e., 20 ms) are available from several providers to increase productivity, they

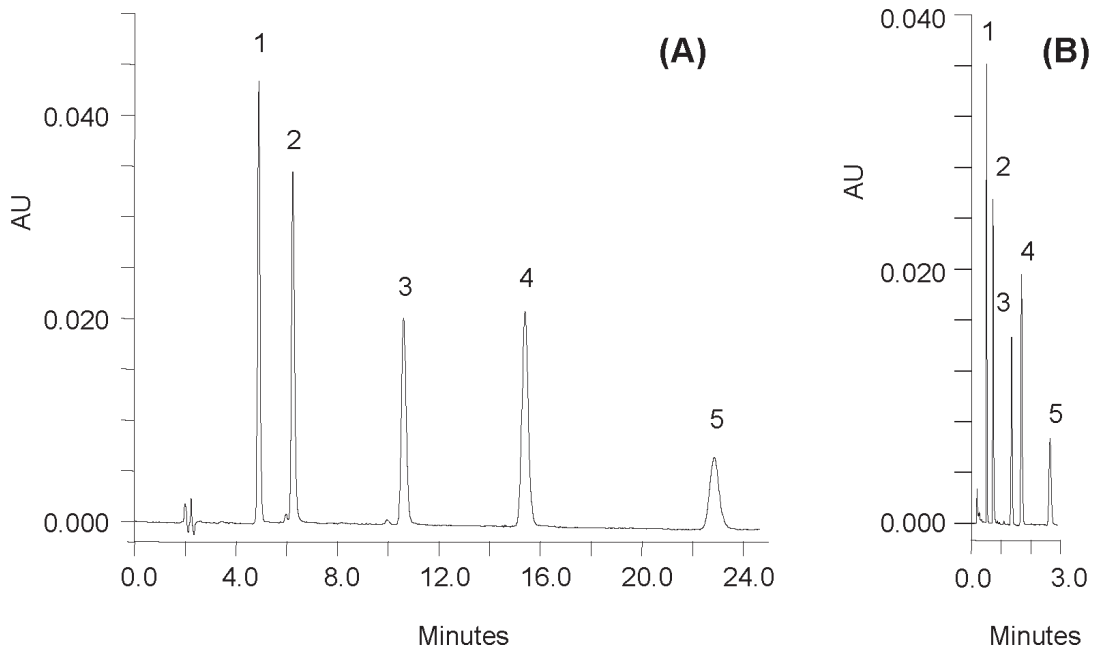

Figure 5. Comparison of chromatograms of mixed standards obtained from (A) HPLC and (B) UHPLC. HPLC conditions: column Kromasil (C18: 250 × 4.6 mm id, 5 μm; Varian, Walnut Creek, CA), mobile phase phosphoric acid 0.1%–MeOH (15 + 85), temperature 25°C. UHPLC conditions: column Waters Acquity (BEH C18: 50 × 2.1 mm id, 1.7 μm), mobile phase phosphoric acid 0.1%–methanol (31 + 69), temperature 35°C. Compound 1 is aloe-emodin, 2 rhein, 3 emodin, 4 chrysophanol, and 5 physcion. Adapted from ref. 55 with permission of Elsevier (Amsterdam, The Netherlands).

always compromised sensitivity, peak width, and sampling rate in UHPLC and should be avoided (40, 41). Figure 4 demonstrates the use of TOF-MS for selective detection and rapid online characterization of natural products, not possible with UV or ELS detectors. As shown in the display of the UHPLC/ESI-positive ion (PI)-TOF-MS trace of the ion *m/z* 741, it permits selective detection of this compound in the crude extract of *Arabidopsis thaliana*. On the other hand, the corresponding spectrum of compound F enabled precise determination of the molecular formula ($C_{33}H_{41}O_{19}$) of its protonated molecule $[M + H]^+$ (*m/z*: 741.2242) and identification of this compound as a flavonol triglycoside.

Fast UHPLC Separations for Quality Assessment of Plant Samples

Plants used, for instance in phytomedicine production, possess some unique properties. For this type of phytopreparation, it is worth mentioning that i) plant extracts are complex and consist, among other things, of numerous metabolites acting synergistically that could hardly be considered separately (42)—some authors even consider that the full herbal product could be regarded as the active compound (14); ii) active compounds are frequently unknown; iii) identity confirmation of the raw material is needed; and iv) composition and concentration of active or toxic compounds in the extract depend on season, time, place of harvest, and extraction. Thus, a suitable standardization and QC procedure is required to guarantee the botanical identity and the quality, safety, and efficacy of the final phytopharmaceutical products. To a lesser extent, because of looser regulation, the same is also valid for dietary supplements or functional foods.

Because of these characteristics, QC of plant extracts is difficult but mandatory (43), and two different techniques can be used. On one hand, classical QC analyses are targeted, aiming to quantify one or a few known pharmacologically active compounds or marker substances, when the active compounds are unknown. These markers are suitable for analytical purposes, but in most cases they have not been validated by activity tests (43). On the other hand, untargeted QC analyses, using a chromatographic fingerprinting approach, provide a wide or complete picture of an herbal product. This second method has recently become an increasingly popular approach for QC and standardization of phytomedicines (42, 44), considering that the full herbal product could be regarded as the active compound (14), and may also be used for chemotaxonomic studies.

Almost all chromatographic or electrophoretic techniques could be suitable for both targeted and untargeted methods. However, only UHPLC methods will be discussed in the present review. For additional information about fingerprint QC analysis, readers can refer to two comprehensive reviews by Liang et al. (13, 14). Recent UHPLC applications for both

targeted and untargeted QC of plant samples have been summarized in Table 3 and will be discussed below.

Targeted QC

Generally, targeted QC consists of high-throughput methods because only a few constituents, representative of the plant sample, have to be evaluated. In this respect, UHPLC is a technique of choice for fast analysis. As previously discussed, it is theoretically possible to obtain a reduction of analysis time by a factor of 9 while maintaining equivalent performance, as experimentally reported in the literature (Figure 2C). Because of the use of different stationary phase chemistries, method transfers in plant analysis are seldom purely geometric. Thus, analysis time is often shorter or longer than the predicted 9-fold reduced time.

For instance, Wang et al. (55) proposed a UHPLC-DAD approach for the simultaneous determination of five anthraquinone derivatives in three *Rheum* species-based medicines. The method has been fully validated in terms of precision, accuracy, and linearity according to International Conference on Harmonization guidelines. The original HPLC method was transferred to UHPLC using the basic rules of method transfer, enabling the new conditions to be used. From a 24 min separation with a 250 × 4.6 mm id, 5 μm column, the analysis time for HPLC was cut to only 1 min using a 50 × 2.1 mm id, 1.7 μm column. As this direct method transfer did not provide a satisfactory separation of two critical compounds, conditions were further optimized by varying the column temperature, flow rate, mobile phase composition, and gradient time. After optimization, a 3 min method was proposed, changing the column temperature from 25 to 35°C and decreasing the flow rate from 1 to 0.75 mL/min, considering the backpressure. As shown in Figure 5, the final UHPLC run time was reduced by 8-fold compared to conventional HPLC, with a new method comparatively more efficient though the resolution and number of theoretical plates was slightly lower. Similar conclusions were drawn by Avula et al. (38), who developed a new UHPLC method for triterpenoid and isoflavonoid identification and quantification in rhizomes of *Actaea racemosa*. Two existing HPLC methods of 35 and 80 min were reduced to 7 min using a 45–65% acetonitrile–methanol (7 + 3) gradient and UHPLC technology. The final method gave shorter analysis time while maintaining good resolution compared to HPLC, saving money and being more environmentally friendly (lower organic solvent consumption). Finally, even faster separations were developed. For example, Götz et al. (46) proposed a powerful 1 min UHPLC-DAD quantification method of *N*-acyl-D/L-homoserine lactones in *Hordeum vulgare* and in *Pachyrhizus erosus* plants with a specific sample preparation.

Only a few applications have been discussed in this section, but the reader can refer to Table 3 for additional information on UHPLC-targeted QC methods. To summarize this table, the standard method aims to quantitatively determine a few known constituents. Separation is quite short, generally 3 to 7 min, on a 50–100 mm UHPLC column in the gradient mode, with acetonitrile as an organic modifier. The detector of choice is generally PDA or quadrupole operating in the MS or MS/MS mode, the latter limiting sample preparation because of the additional selectivity provided by MS.

Except for efficiency and analysis time, it is important to consider selectivity when developing a UHPLC method. Developing a fast separation can be tricky and time-consuming when numerous chromatographic parameters need to be optimized simultaneously. In order to quickly and efficiently develop a UHPLC method, Li et al. (53) proposed methodology for the determination of 10 diterpenoids in *Salvia miltiorrhiza* using a central composite design approach (i.e., experimental design). This method development strategy can be considered as generic and applicable to any other plant extract. Their methodology consists of varying the most relevant chromatographic parameters, i.e., gradient time, column temperature, and flow rate, and finding their optimal values. For this purpose, retention time of the most retained compound and the most critical resolution were considered. In their specific example, Li et al. (53) changed the initial conditions, i.e., 10 min chromatographic run without adequate peak separation, into a satisfactory separation within 8 min. Such an optimizing tool is highly interesting in method development, but it is worth noting that HPLC modeling software would be even more powerful than such experimental design and would be less time-consuming. However, both approaches are still scarcely used for plant extract analysis at present.

Untargeted QC

Unlike the methods described above, untargeted QC is not intended to quantify a few markers, but to qualitatively compare fingerprints without peak assignment. Quantification can take place only in a second step. Generally, analysis time is longer than for targeted QC because of the sample complexity but remains acceptable, i.e., less than 15 min, since UHPLC is used. Sample preparation is unselective, and PDA or TOF-MS operating in high-resolution mode on the full mass range are considered the detectors of choice. A list of the untargeted QC applications in UHPLC is provided in Table 3.

Because a wide range of metabolites should be analyzed during chromatographic fingerprinting, sample preparation should be adapted. For instance, Liu et al. (64) suggested a two-step sample preparation for investigating the root of *S. miltiorrhiza*. Two distinct extraction procedures were considered, the first one performed with 10% methanol (MeOH) for the extraction of most hydrophilic components and the second one with 90% MeOH for the most lipophilic compounds. Then, both extracts were mixed together in the ratio 1:4 to obtain a well-balanced fingerprint. The UHPLC fingerprinting analysis was carried out on a 100 × 2.1 mm id, 1.7 μm UHPLC column at 30°C using the following acetonitrile (ACN; A)—phosphoric acid (0.1%; B) gradient: 10–25% A in 5 min, 25–50% A in 5 min, then 80% (A) for 6 min. The flow rate was set to 0.5 mL/min. Compounds were detected by a UV detector set at 280 nm.

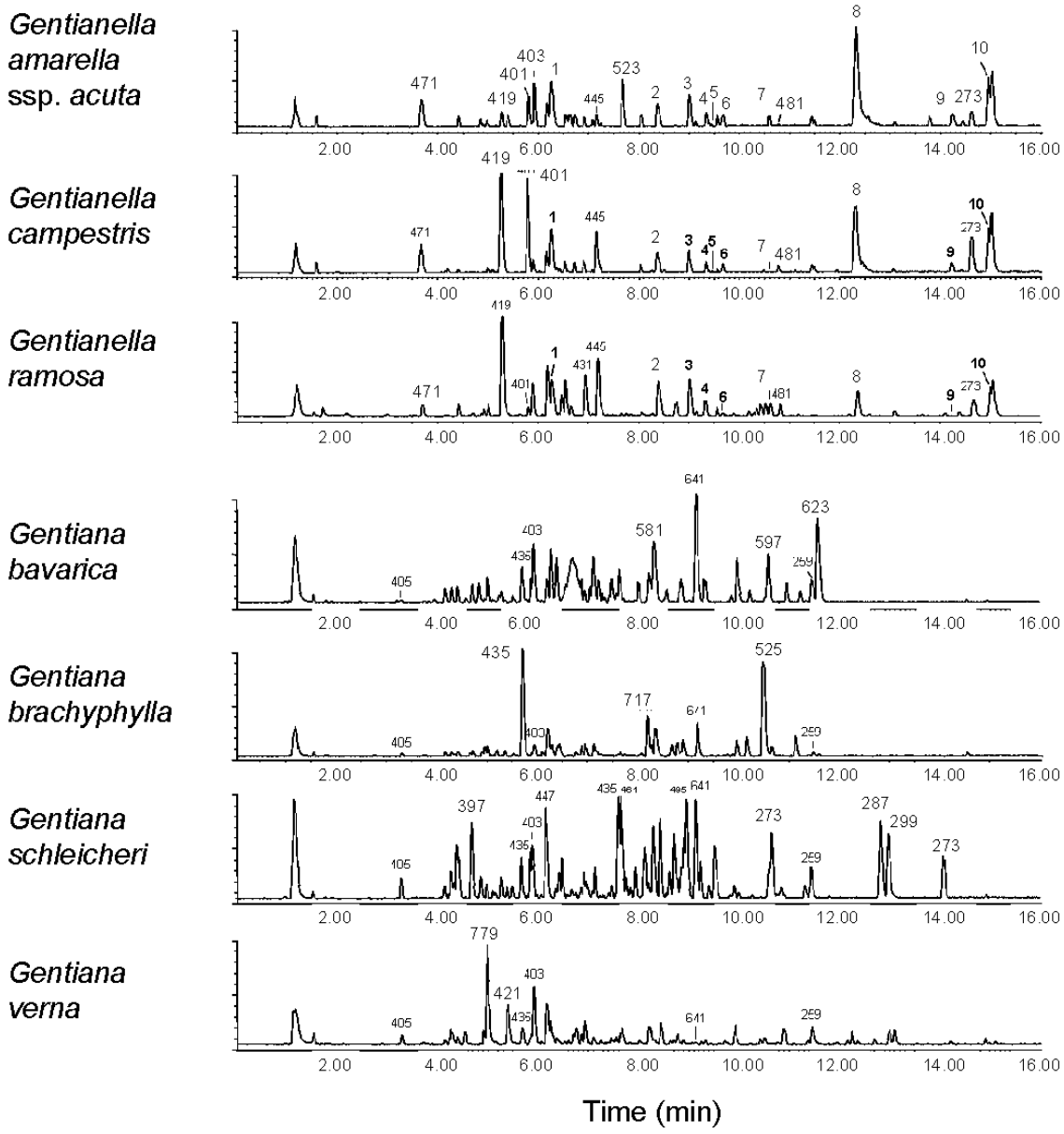

Figure 6. Chromatographic fingerprints of extracts of different *Gentiana* and *Gentianella* in a chemotaxonomic study. UHPLC conditions: 150 × 2.1 mm id, 1.7 μm UHPLC column, gradient 5–55% ACN within 15 min, detection: TOF-MS. Adapted from ref. 96 with permission of Wiley.

This 16 min UHPLC analysis in conjunction with the two-step extraction provided a powerful fingerprinting QC covering markers and unknown compounds. This approach used the same tools as in metabolomic analysis and may be explored in another way, as shown by Pongsuwan et al. (65), who used a fingerprinting strategy to study the correlation between different green tea grades and their chemical composition. In their work, 56 different teas were evaluated and analyzed by UHPLC/TOF-MS with minimal sample preparation. Separation was carried out on a 150 × 2.1 mm id, 1.7 μm UHPLC column at 40°C, with a 0–55% ACN in water (both with 0.1% formic acid) gradient over 10 min. After appropriate multivariate data analysis [i.e., principal components analysis (PCA) and partial least-squares], teas could be ranked and higher-grade teas were clearly separated on the PCA projection.

An untargeted QC by fingerprinting provides significantly more information than a targeted QC focused only on a few biomarkers. This type of approach is particularly useful when the active ingredient(s) of a given extract could not be clearly defined and the QC relies on the total composition. The main limitation of untargeted QC is the long analysis time (i.e., around 1 h) for routine use of the method (64). However, since it is possible to decrease

analysis time by 5–10 times with UHPLC instrumentation, and because high acquisition rate analyzers are available, it is likely that fingerprinting analysis will be increasingly used for QC analysis of plant extracts.

It is finally worth mentioning fingerprinting as a chemotaxonomic tool to discriminate plant species based on the chromatographic profile. For example, Urbain et al. (96) applied UHPLC/TOF-MS to distinguish *Gentiana* and *Gentianella* genera among the Gentianaceae family based on their secondary metabolite content. Separations were carried out on a 150 × 2.1 mm id, 1.7 μm UHPLC column in 15 min with a 5–55% aqueous-ACN gradient. The TOF-MS detector provided high mass accuracy and resolution and allowed determination of the elemental composition of the compounds for dereplication purposes. Xanthones, flavonoids, and secoiridoids were identified by this means. As shown in Figure 6, the fingerprints of the three *Gentianella* species were strikingly similar. On the contrary, fingerprints of *Gentiana* species were very different from those of *Gentianella* and from each other. Several compounds were unique to each genus and, therefore, could be used as biomarkers.

Fast and High-Resolution UHPLC Experiments for Plant Metabolomics

The term "metabolomics" corresponds to the analysis of the whole collection of metabolites (i.e., small molecules, MW < 1000 g/mol) that participate in general metabolic reactions of organisms such as plants, mammals, or humans. The size of the metabolome can vary from several hundreds of metabolites for simple organisms such as yeast to 200 000 primary and secondary metabolites for the most complex plant samples, and the human metabolome can be expected to be even larger. In addition, metabolites constitute a very diverse set of atomic arrangements that provides heterogeneous chemical properties. Finally, the metabolome extend over an estimated 7–9 magnitudes of concentration (100). Because of this inherent complexity, it is extremely difficult to analyze all metabolites in a single analysis; therefore, two complementary approaches are mainly used for the determination of new biomarkers in metabolomics, namely, metabolic fingerprinting and metabolite profiling. For the former, the aim is to quickly compare patterns of metabolites to provide sample classification, without any quantification and metabolic identification. On the contrary, metabolic profiling focuses on a limited number of predefined metabolites, and analytical methods are specifically developed for their determination (identification and quantification; 7).

Because of the complexity of crude plant extracts and since metabolites can be found at very low concentrations, the use of analytical systems providing high resolution and sensitivity is recommended. In this context, the use of UHPLC in conjunction with TOF-MS detection is certainly the tool of choice and has been used for a few years for plant metabolomics. For instance, Dan et al. (70) and Xie et al. (76, 77) reported the metabolic fingerprinting of various medicinal *Panax* herbs, while our laboratory implemented a generic UHPLC/TOF-MS platform for the fingerprinting, profiling, and targeted analysis of metabolites in NP extracts. Our strategy was mainly applied for the analysis of the model plant *A. thaliana* (28, 34, 41, 68, 72–74).

Metabolic Fingerprinting: Application to Panax Herbs

In a series of papers, Xie et al. (76, 77) reported their metabolomic investigations of *Panax* herbs for the rapid differentiation and identification of complex traditional Chinese medicine (TCM) extracts. In a first study, it was shown that five different *Panax* herbs: *P. ginseng* (Chinese ginseng), *P. notoginseng*, *P. japonicus*, *P. quinquefolium L.*, and *P. ginseng* (Korean ginseng), cultivated in different locations in Asia, could be differentiated based on their metabolite profiling (76). The appearance and some ingredients of these plants are quite similar, but their pharmacological activities are obviously different because of the variation in the nature and quantity of saponins in each herb (77). To identify the variations in bioactive components among different *Panax* herbs, a UHPLC quadrupole (Q) TOF-MS procedure was employed in conjunction with the unsupervised pattern recognition method, PCA. The 20 min gradient used permitted an obvious differentiation of the various *Panax* samples based on the presence or absence of several chemical markers; 25 saponins were tentatively identified using the high mass accuracy of the QTOF-MS analyzer and verified with available reference standards (76). In comparison, only 11 saponins were identified by conventional HPLC with a run time of 80 min. However, this result could not only be attributed to the use of UHPLC versus HPLC, but to the detector technology used in both studies; a powerful QTOF-MS instrument was used with UHPLC while a single- quadrupole instrument was used with conventional HPLC. According to the authors, this methodology can be applied to different plants and/or plants from different geographical locations as it is generic. Using a similar procedure, Dan et al. (70) also demonstrated the possibility to discriminate various parts of *P. notoginseng*, including the composition of flower buds, roots, and rhizomes. The analytical procedure was identical and consisted of UHPLC/MS followed by PCA. The chemical biomarkers responsible for differentiation were again saponins (i.e., ginsenosides) and have been identified by an ESI-PI-QTOF-MS analyzer.

A very similar procedure was used by Chan et al. (67), who investigated the differences between *P. notoginseng* in the raw and steamed forms, both possessing very different pharmacological properties. The raw form is generally used in TCM to treat cardiovascular diseases, while the steamed form is used as a tonic to treat anemia. Again, PCA analysis of the UHPLC/TOF-MS fingerprints provided a good discrimination of slight variations recorded within the same plant species due to different geographical locations, cultivations, and collection times.

The procedure previously applied to *Panax* herbs was further extended by Sawada et al. (82), who proposed a

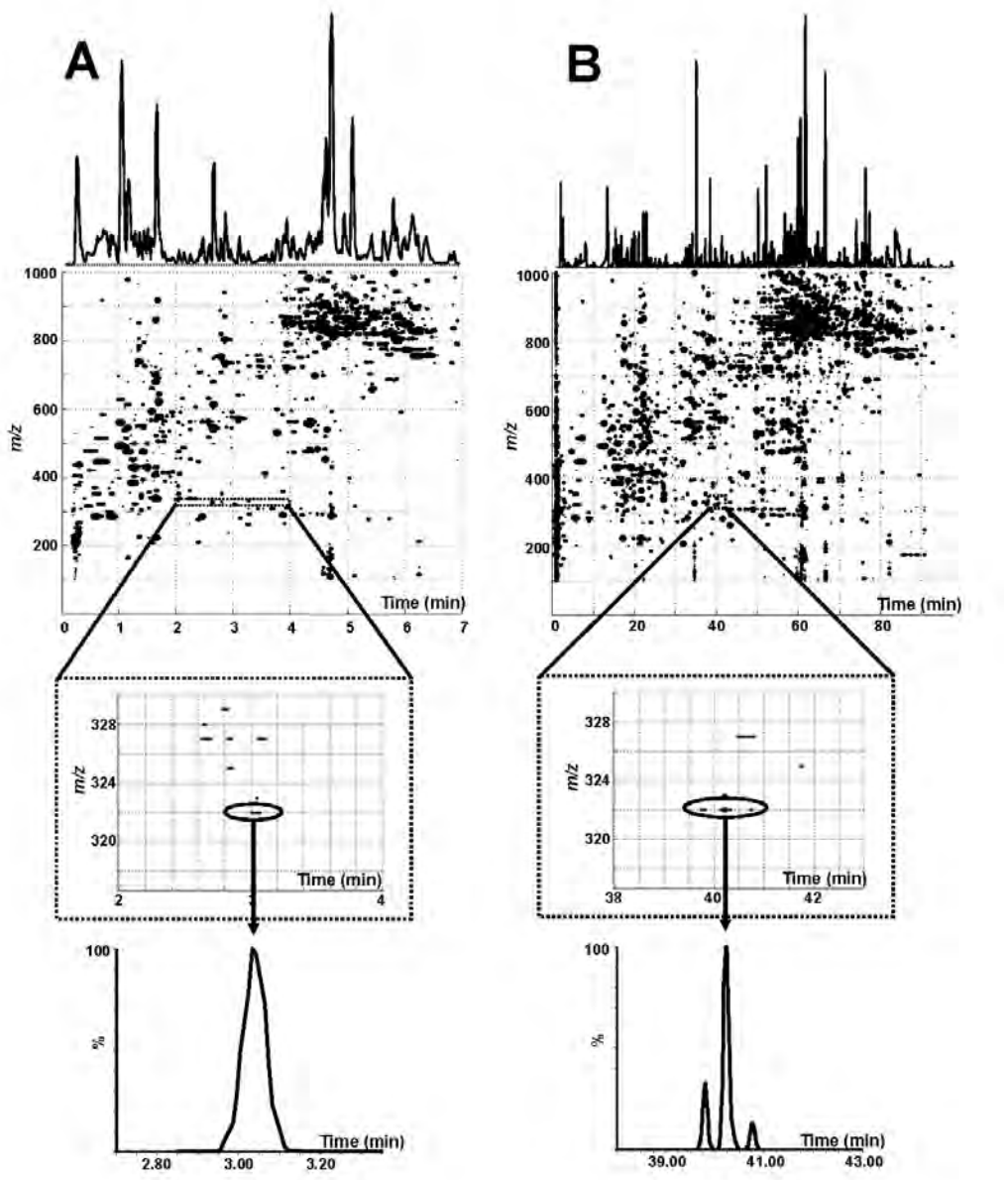

Figure 7. UHPLC/TOF-MS analysis of an *A. thaliana* extract in the form of three-dimensional ion maps. (A) Metabolic fingerprinting using a 7 min gradient time and a 50 × 1.0 mm id, 1.7 μm column at 300 μL/min; (B) metabolite profiling using a 97 min gradient time and a 150 × 2.1 mm id, 1.7 μm column at 300 μL/min. The insets correspond to the separation of *m/z* 322.20 isomers obtained with both UHPLC conditions.

practical metabolomics methodology based on UHPLC/MS-MS for quantifying hundreds of important targeted metabolites in various plant samples with high throughput. In a first step, an MS/MS database for about 500 standard metabolites was constructed. Thus, a generic UHPLC/MS/MS method with total analysis time of only 3 min was developed for the determination of these 500 metabolites. Then, in a second step, the strategy was applied to various biological samples extracted from different plants belonging to Brassicaceae, Gramineae, and Fabaceae. A hierarchical cluster analysis was finally used to assess differences among the plant families. This strategy is very promising and is practically applicable for large-scale comparative metabolomics.

Multistep Strategy for Fingerprinting, Profiling, and Target Analysis: Application to A. thaliana

We investigated a sequential strategy (i.e., metabolic fingerprinting, metabolite profiling, and metabolite target analysis) for the detection, isolation, and identification of biomarkers induced by stress in the model plant *A. thaliana*, after leaf-wounding, which mimicked herbivore attack. Because the proposed approach is generic, this analytical platform could be used to screen various other plant extracts without further reoptimization.

Initially, a high-throughput fingerprinting (i.e., 7 min analysis time; Figure 7A) was carried out to discriminate unwounded and wounded samples by UHPLC/TOF-MS using

a short column of 50 × 1.0 mm id eluted at an elevated flow rate, 300 μL/min (28). High-quality LC/MS data were obtained thanks to excellent detection sensitivity and low retention time variability. Such an analysis was required because specimens from different days of cultivation were considered, thus increasing the metabolic variations. Data treatment was then applied to form adequate pooled samples having a common metabolic pattern within groups, while avoiding wrong interpretations due to samples having atypical behavior.

In a second step, longer UHPLC columns (150–300 × 2.1 mm id) operating at low mobile phase flow rates (200–300 μL/min) were used for high-resolution metabolite profiling of selected pool samples (Figure 7B; 28, 74). Because the column phase chemistry remained identical between the fingerprinting and profiling steps, the high- throughput method was easily transferred to high-resolution profiling using basic rules for method transfer in LC (24, 25), and analysis times were extended up to 100 and 300 min for the 150 and 300 mm column length, respectively. To maintain detailed metabolite profiling of crude plant extracts while avoiding very long analysis times, we recently proposed increasing mobile phase temperature in UHPLC (34, 41) in order to work with a higher flow rate, because of the viscosity and generated backpressure decrease with temperature. As expected from theory, the analysis time was reduced by 2- to 3-fold at 90 versus 30°C for profiling plant extracts such as *A. thaliana* that contain metabolites spread over a large polarity range. In addition, the stability of NPs under high temperature conditions was investigated, and no apparent degradation was evidenced for a representative mixture of NPs and for the different metabolites detected in selected plant extracts. This second step (i.e., high-resolution metabolite profiling) allowed confirmation of the presence of different biomarkers and was important to avoid coelution problems associated with the convoluted nature of the extract, i.e., complex separation of closely related isomers, as shown in the insets of Figure 7 and as also reported elsewhere (87), and to validate the molecular mass of the different stress-related compounds (28, 74). Some of the most important biomarkers were easily identified based on their molecular formula, additional pseudo MS/MS experiments using collision-induced dissociation (93), and comparison with standards. They were known signaling molecules such as jasmonic acid and other related oxylipins. Other biomarkers were unknown and could not be identified based on UHPLC/MS data only.

The final step of the process consisted of the complete structural elucidation of minor biomarkers using LC/MS-triggered preparative isolation and capillary NMR spectrometry (capNMR) at the microgram scale (74). Because of the complex nature of plant extracts, the purification of metabolites at low concentrations is a challenging task. For this part of the work, the high-resolution UHPLC profiling method was transferred to the semipreparative scale by using a 10 mm column id packed with 5 μm particles of the same material. Due to the very low concentration of biomarkers, a baseline resolution was needed to ensure sufficient purity for the capNMR analysis. Thus, two semipreparative columns of 250 mm length were coupled in series to attain sufficient efficiency (i.e., around 30 000 theoretical plates). This strategy was applied for the purification and identification of known signaling molecules, as well as original oxylipins and jasmonates, using a capNMR probe. In addition, minor and closely related isomers such as the four hydroxylated forms of jasmonic acid, sharing an identical molecular formula and fragmentation pathways, were baseline-separated and identified with microflow NMR spectrometry. The accumulation profiles of these positional isomers in *A. thaliana* were investigated based on metabolomic data for different wound time points, and revealed a delay accumulation compared with jasmonic acid (72). This demonstrated that these hydroxylated derivatives were probably clearance metabolites of jasmonic acid and provided new insights in jasmonate biochemistry. Other new polar jasmonates were efficiently identified by this means (72, 84), and the study of their expression profile provided numerous new results on jasmonic acid metabolism (72). Furthermore, tissue-specific studies (local versus systemic leaf metabolomics) also revealed new insights on long-distance signaling (72, 101).

Janson et al. (80) proposed a more comprehensive global metabolomic approach in which both participating organisms in a plant-insect herbivore interaction were chemically analyzed to gain more insight into the metabolites possibly involved in such an interaction. Their study analyzed the interaction between feral cabbage (*B. oleracea*) and small caterpillars (*Pieris rapae*) using a 15 min run time in UHPLC/TOF-MS. It was concluded that the attack history of *Brassica oleracea* plants affects a specific part of the *P. rapae* metabolome. Other UHPLC-based metabolomic studies are summarized in Table 3.

Conclusions

As shown in this review, UHPLC is beginning to gradually and advantageously replace conventional HPLC methods in various fields of plant analysis. The number of applications is, however, still small compared to HPLC. In some research fields, such as metabolomics, the technique provides clear advantages in terms of reproducibility, resolution, and throughput that could not be attained by conventional HPLC methods in practically achievable analysis times. Such characteristics are essential for a satisfactory comparison of fingerprints with data-mining methods and for a very precise localization of related biomarkers. In all other examples discussed, the efficiency of UHPLC—either in terms of high throughput (QC and fingerprinting) or high resolution (dereplication and profiling)—represents clear advantages.

One of the drawbacks of the technology is that because of the small (sub-2 μm) particles used in columns, dedicated LC instrumentation is required to cope with the high backpressure generated. However, as shown, many manufacturers now provide systems that handle very high ($\Delta P_{max} \geq 1000$ bar) or intermediate (500 bar < ΔP_{max} < 800 bar) pressure that can accommodate such conditions. Another limitation was that

the number of phase chemistries was rather restricted at the beginning of UHPLC. However, at present, a great choice of columns with enhanced performance is available, and almost any type of separation previously performed by HPLC can be transferred to UHPLC.

An alternative to sub-2 μm particles for working with low backpressure resides in the recent development of columns with Supelco Fused-Core™ particles consisting of a 1.7 μm solid core surrounded by a 0.5 μm porous silica shell (Sigma-Aldrich, St. Louis, MO). Due to the reduced mass transfer path length and narrow particle size distribution, these particles have demonstrated similar chromatographic performance compared to sub-2 μm particles (102). Their backpressure, however, is what would be expected for 2.7 μm particles, more than 50% lower than the sub-2 μm counterpart at the same mobile phase velocity. Such columns have been recently successfully applied to complex mixtures of NPs with very similar structures, MWs, and functional groups, and their performance was close to that achieved in UHPLC with sub-2 μm particles (103). Fused-Core™ particles can thus be a good low-pressure alternative to columns packed with sub-2 μm particles for separation of complex mixtures with only a small sacrifice in peak efficiency.

Further development for the improvement of the resolution of complex mixtures of NPs would be the implementation of two dimensional (2D)-LC (104) to complement the resolving power of UHPLC. One strategy could be to use UHPLC as a fast method in the second dimension of a 2D-LC setup. HILIC and RP-LC strategies, which are two orthogonal approaches that can be carried out with RP solvent systems, could, for example, be used in the first and second dimensions, respectively. The efficient coupling of LC orthogonal methods still represents an important challenge for which UHPLC, thanks to its fast separation capabilities, can play a strategic role.

As has been discussed, the fast separations obtained in UHPLC are challenging for the detector, and very high acquisition rates are needed to cope with this issue. At present, the new generation of TOF-MS instruments has been designed to be compatible with such elevated acquisition frequencies. However, other very high-end MS instruments that can generate important online structural information, such as orbitraps, need further improvement to be compatible with UHPLC while keeping their full resolution power.

From its introduction for NP analysis in the early 1980s, HPLC has represented an important breakthrough. The recent development of UHPLC similary represents a key evolution of the technique that gives to NP chemists the possibility of crude extract analysis in a much more detailed manner with higher efficiency. With the increasing requirements for QC, profiling and fingerprinting, dereplication, and metabolomics, the demand for hyphenated systems that combine the best achievable speed and resolution of both the chromatographic and spectroscopic components will increasingly continue and, in this context, UHPLC represents a very valuable tool.

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