Study of the composition of biologically active fractions from plant extracts by advanced techniques of HPLC and UHPLC/MS

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Abstract
L'analyse d'extraits de plantes a augmenté d'importance soit pour la recherche des nouvelles molécules pharmaceutiques que pour le contrôle qualité des médicaments. En analytique, la récente tendance est de réduire les temps d'analyse afin d'augmenter la productivité, mais pour les analyses des plantes, les nouvelles techniques (hautes températures, particules sub-2µm ou particules superficiellement poreuses sub-3µm) sont utiles plutôt pour augmenter l'efficacité chromatographique et la séparation. Pour développer un nouveau méthode analytique, il est obligatoire de déterminer la meilleure phase stationnaire (chimie et dimensions) et phase mobile (solvent organique, pH et température). Pour évaluer l'efficacité de 5 colonnes core-shell et sub-2 µm, trois extraits ont été sélectionnés: le romarin, le genévrier et le ginkgo. La capacité de pics est le paramètre utilisé pour comparer les performances des colonnes. Celle-ci varie entre les colonnes par rapport aux différents extraits, mais les meilleures restent l'Ascentis (colonne core-shell) et Acquity (colonne sub-2µm). Pour augmenter les […]

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Study of the composition of biologically active fractions from plant extracts by advanced techniques of HPLC and UHPLC/MS.

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Abstract

Plant analyses have become more and more important both for the search of new drug molecules and for more and stricter controls on plant medicines. Every plant extract consists of several components belonging to different chemical classes. It is therefore essential to have tests and methods able both to discriminate compounds with very close features and classes of compounds with different polarities in a reasonable analysis time. Liquid chromatography (LC) has proved to be the best technique to analyze non-volatile molecules.

Recent trends suggest reducing time and increasing throughput. Several solutions have been used to reach this goal, such as high temperature, sub-2-μm and core shell particles, monolithic columns and sensitive detectors. All these new technologies are very useful especially in increasing efficiency and compound separation, while shorter analysis time is only a secondary effect.

To develop a new method to analyze plant extracts the best conditions of stationary phase, chemistry and dimensions of the column, organic solvent, pH of the mobile phase and temperature are key. Stationary phase chemistry is one of the most important parameter for the selectivity, but several different chemistries are required to notice that. pH variations are particularly useful for compounds with ionisable centers on the molecule. It is very useful to work with neutral compounds, since they are retained for longer on the column making it easier to reach a good separation. Temperature has a weaker effect on selectivity compared to pH or proportion of the organic solvent variations, but it is useful to decrease backpressure, leading both to work at higher flow rates and to a reduction in analysis time.

Three plant extracts with increasing complexity were chosen to evaluate the efficiency of core-shell and sub-2 μm columns, namely rosemary, juniper and ginkgo. Peak capacity was chosen as a parameter to evaluate the efficiency of the different columns. It varies between the columns in regards to the different extracts, although Ascentis, a core-shell column, and Acquity, a sub-2 μm proved to be the best columns. Differences in selectivity and retentions were highlighted among the five columns, even if they have the same C18 stationary phase. To increase efficiency further without using very sensitive detectors, but a conventional UV detector, column lengths were gradually increased coupling together up to three columns. The column of choice fell on the Acquity, because it both gave a very high peak capacity and was able to withstand up to 90°C, while core-shell were limited to 60°C.

Finally, conventional methods used both to identify with MS detection and to quantify markers present in the absolute extract were optimized on a performing UHPLC system exploiting both columns with smaller diameters and core-shell column. The result was a partial reduction of analysis time, but more importantly, it was the better separation and the reduction of co-elutions of the analysis.

An alternative technique, supercritical fluid chromatography (SFC), was tested to verify if it is possible to substitute LC with other greener techniques.
**Résumé**

L'analyse d'extraits de plantes prend de plus en plus d'importance soit dans le but de rechercher des nouvelles molécules pharmaceutiques ou alors pour le contrôle qualité des médicaments dérivés de plantes, ces derniers étant de plus en plus stricts. Les extraits des plantes sont formés de nombreux constituants qui peuvent également appartenir à différents classes chimiques. Les méthodes analytiques doivent donc être capable de discriminer à la fois des composés avec des propriétés très proches mais aussi des classes de composés ayant des polarités différentes, le tout dans un temps d'analyse raisonnable.

La chromatographie liquide (LC) est la technique de choix pour analyser des molécules non-volatiles.

La tendance récente dans le domaine analytique est de réduire de plus en plus les temps d'analyse afin d'augmenter la productivité. De nombreuses solutions peuvent être utilisées pour atteindre ce but, tels que l'utilisation de hautes températures, de particules poreuses sub-2µm, de particules superficiellement poreuses (core-shell) sub-3µm, ou enfin de supports monolithiques. Pour les analyses des plantes, des nouvelles techniques sont utiles mais plutôt dans le but d'augmenter l'efficacité chromatographique et la séparation des composés. La diminution du temps n’est que un paramètre secondaire avec des échantillons complexes.

Pour développer une méthode analytique adaptée aux extraits des plantes, il est obligatoire de déterminer la meilleure phase stationnaire (chimie et dimensions), et phase mobile (type de solvant organique, pH et température). La chimie de la phase stationnaire est un des paramètres les plus influents pour changer la sélectivité, et il est donc important d'avoir à disposition différentes phases stationnaires. Une variation du pH est également particulièrement efficace pour les molécules présentant des groupes ionisables. La température a un impact plus limité sur la sélectivité par rapport aux changements de pH et solvant organique, mais la température reste un paramètre utile pour diminuer la pression dans la colonne et donc pour travailler à des débits plus importants afin d'obtenir des temps d’analyses plus courts.

Pour évaluer l'efficacité de 5 colonnes core-shell et sub-2 µm, trois extraits des plantes de difficulté croissante ont été sélectionnés : le romarin, le genévrier et le ginkgo. La capacité de pics est le paramètre utilisé pour comparer les performances de différents types de colonnes. Celle-ci varie entre les colonnes par rapport aux différents extraits, mais les meilleures colonnes restent l’Ascentis (colonne remplie de particules core-shell sub-3µm) et Acquity (colonne remplie de particules poreuses sub-2µm). Des différences de sélectivité et rétention ont été mises en évidence entre les cinq colonnes testées, malgré que toutes les phases stationnaires étaient greffées avec des groupements C18). Pour augmenter encore plus les performances chromatographiques, la longueur de la colonne a été graduellement augmentée jusqu’à obtenir une longueur totale de colonne égale à 45 cm (trois colonnes remplies de particules sub-2 µm couplées en série). L'utilisation d'une température de phase mobile plus élevée à également été considérée, afin de travailler dans des conditions de pressions acceptables. Enfin, cette méthodologie a été utilisée pour des applications qualitatives ou quantitatives, tel que la détermination des constituants présents dans l’absolu de peuplier.

Pour finir, une technique alternative, la chromatographie en phase supercritique (SFC), a également été étudiée afin d’évaluer une possible substitution avec la LC.
**Riassunto**

Le analisi di campioni vegetali stanno diventando sempre più importanti sia per la ricerca di nuovi principi attivi, che per i controlli più rigidi richiesti per i fitoterapici. Ogni estratto vegetale è formato da un gran numero di componenti, che possono anche appartenere a diverse classi chimiche. È quindi necessario che i metodi analitici utilizzati siano sia in grado di discriminare composti con caratteristiche molto simili che riuscire a ottenere una separazione in tempi ragionevoli di classi di composti con polarità diverse.

Per campioni poco volatili la cromatografia liquida (LC) rimane la tecnica d’elezione. Recentemente il trend più seguito in ambito analitico è di ridurre sempre più il tempo di analisi in modo da aumentare la resa. Diverse soluzioni sono state sviluppate per raggiungere questo scopo: colonne impaccate con particelle sub-2 µm e core-shell, colonne monolitiche, alta temperature e detector con alta sensibilità. Per analisi di estratti vegetali tutte queste nuove tecnologie sono utili soprattutto per aumentare l’efficienza e la separazione dei composti e solo come effetto secondario per ridurne il tempo di analisi.

Per sviluppare un metodo ottimizzato per estratti vegetali è fondamentale trovare le condizioni più favorevoli di fase stazionaria e dimensioni della colonna, fase organica e pH della fase mobile e temperatura. La fase stazionaria è uno dei parametri che influenza maggiormente la selettività in un’analisi, ma bisogna disporre di colonne con fasi stazionarie di diversa ritentività. Variazioni di pH risultano essere particolarmente utili per quelle molecole che presentano centri ionizzabili, è infatti importante che queste non siano cariche, in modo da essere più ritenute. La temperatura non ha effetti evidenti nella selettività come il pH o la variazione della percentuale di fase mobile, ma è utile per diminuire la contropressione, permettendo quindi sia flussi di lavoro maggiori, che riduzione del tempo di analisi.

Per valutare l’efficienza delle colonne core-shell e sub-2 µm sono stati scelti tre estratti vegetali di diversa complessità: il rosmarino, il ginepro e il ginkgo. La *peak capacity*, utilizzata per valutare l’efficienza separativa delle colonne, varia in relazione al campione usato, anche se le colonne Ascentis e Acquity risultano sempre essere le più performanti. Alcune differenze di selettività e ritenzione sono state evidenziate fra le diverse colonne testate, anche se tutte presentano la medesima fase stazionaria C18. Per accrescere ulteriormente l’efficienza separativa senza usare detector maggiormente sensibili, ma un convenzionale detector UV, la lunghezza della colonna Acquity è stata progressivamente aumentata accoppiandone tre in serie e la temperatura aumentata a 90°C. La scelta è caduta su colonne sub-2 µm sia per le ottime prestazioni separate dimostrate precedentemente sia perché, a differenza delle core-shell, sopportano una temperatura fino a 90°C.

Infine il metodo usato in HPLC convenzionale per analizzare e quantificare composti presenti nell’assoluta di pioppo, che è una matrice complessa, è stato ottimizzato su un sistema UHPLC sfruttando una colonna core-shell con diametri ridotti. Il risultato ottenuto è sia una diminuzione parziale del tempo di analisi, ma soprattutto una migliore separazione e riduzione delle coeluizioni nella parte centrale dell’analisi.

Per terminare, una tecnica alternativa, la cromatografia a fluidi supercritici, è stata studiata per valutare una possibile sostituzione con LC.
1. Introduction
Phytochemical analysis is a very important field in phytochemistry to determine the quality and eventually quantitative composition of plant material. The goal of these analyses is to find the metabolites that possess pharmaceutical or nutritive functions.

Plants have always been used for therapeutical purposes since the past. Every part of the plant can be exploited in this respect seeds, berries, roots, leaves, barks or flowers. Many health problems can be treated with herbal drugs, such as digestion, dermatological problems, cough, common cold, migraine, premenstrual syndrome, menopausal symptoms, chronic fatigue, mild gastrointestinal disorders, anxiety and sleeping problems. The World Health Organization (WHO) recently estimated that 80% of world population use herbal medicines to take care of their healthcare.

WHO and European and national pharmacopoeias set different parameters for quality control (QC) for both crude drug materials and finished products. For standardization of crude drugs characteristic constituents, known as marker compounds, can be very useful to identify, set the quality of the material, standardize the manufacturing processes or evaluate the stability. These compounds can be chemical markers, which are found among the major constituents in the plant material and can easily be detected, or active markers, which are the chemical constituents that possess the therapeutic activity in the plant. All the information can be obtained by different chromatographic techniques, such as liquid chromatography (LC), gas chromatography (GC), capillary electrophoresis (CE). For non-volatile compounds the most used technique is LC.

Analytical laboratories have recently been involved in new high resolution techniques, also under the pressure of the increasing interest in the “omics” sciences [1,2], that include a huge number of different complexity samples, going from protein tryptic digests to plant natural extracts. The main effort derives from the massive number of compounds that need to be separated in each sample.

Different approaches in plant analyses related to the goal of the analysis are available: metabolic fingerprint, metabolic profiling, dereplication and targeted analysis. Each method is able to highlight different characteristics in the samples, as a consequence approaches have different objectives: acquire a simple profile or identify each single peak.

It is known that resolution (Rs) in chromatography is correlated to three parameters:

- selectivity;
- retention;
- efficiency [3].

The last two factors influence the separation of couples of consecutive critical peaks more strongly. However, in the case of very complex mixtures or fast separations, nonspecific working conditions are preferably chosen, due to the difficulty to control selectivity and retention at the same time. The ideal choice is to apply conditions that maximize the plate number (N), since efficiency also contributes to resolution.

Over the past decades, the need to improve data quality and the increase of sample throughput has generated a considerable interest in fast LC technologies. A number of different approaches has been proposed to improve both efficiency and resolution [4,5]:

- monolithic supports;
- high temperature liquid chromatography (HTLC);
- columns packed with small particle stationary phases (sub-2 µm) suitable to operate under ultra-high pressure conditions (UHPLC);
- superficially porous silica particles (core-shell columns);
- sensitive detectors.

In my work of thesis I focused my attention on HTLC, UHPLC, core-shell columns and sensitive detector to increase efficiency and separation for complex plant analyses.
REFERENCES


2. High efficiency in plant analysis: background
2.1 Evolution in Chromatographic Techniques
2.1.1 Kinetics rules in High Performance Liquid Chromatography (HPLC)

HPLC aims to separate the maximum number of compounds of a sample with the highest resolution in the minimum time.

A chromatographic column can be imagined as a large number of separate layers, called theoretical plates. The separation of the compounds present in a sample occurs between these plates by transferring in the equilibrate mobile phase [1]. The lower these plates, the higher the efficiency of the column. In reality, band spreading ($\sigma^2_{\text{tot}}$) works against this process. This effect is essentially due to two different components: column ($\sigma^2_{\text{col}}$) and extra-column effects ($\sigma^2_{\text{ext}}$) (eq. 1.1).

Equation 1.1: contributions of column and extra-column effects to the total variance.

$$\sigma^2_{\text{tot}} = \sigma^2_{\text{col}} + \sigma^2_{\text{ext}}$$

The column contribution $\sigma^2_{\text{col}}$ is the parameter that cannot be modified as it depends exclusively on the column geometry (characteristics).

The chromatographic system volume $\sigma^2_{\text{ext}}$ derives from the contributions of injector, detector and connecting tubes (eq. 1.2).

Equation 1.2: contributions of extra-column effects to the chromatographic system volume.

$$\sigma^2_{\text{ext}} = K_{\text{inj}} \cdot \frac{V^2_{\text{inj}}}{12} + K_{\text{cell}} \cdot \frac{V^2_{\text{cell}}}{12} + \tau^2 \cdot F^2 + \frac{r^4 \cdot l_c \cdot F}{7.6 \cdot D_m}$$

The injection contribution depends on the injection into the column ($K_{\text{inj}}$) and sample volume. The ideal is to inject:
- a sample dissolved in a solvent weaker than the initial mobile phase conditions,
- a volume between 1 and 5% of the column volume.

Detector contribution depends on the time constant ($\tau$) and flow rate ($F$), while the volume of the cell ($V^2_{\text{cell}}$) is a fixed parameter and it can only be varied by changing it. It is important to set a correct $\tau$ value to avoid peak distortion due to the slow response of the detector, not to lack in separation.

The last contribution is due to tubings and it depends on both their length and radius. Volumes can be reduced by replacing conventional tubings: the new optimized connections should be as short as possible and the diameter chosen in regard of both volumes and generated backpressure. When even with an optimized instrument the extra-volume is still too high, the only solution is to operate with high retention factors ($k$) to increase $\sigma^2_{\text{col}}$ (eq. 1.1).

Column efficiency is commonly described by the number of plate ($N$), that is related to H or HEPT (eq. 1.3).

Equation 1.3: number of theoretical plates ($N$).

$$N = \frac{L}{H}$$

HEPT depends to the linear velocity of mobile phase, $u$, and its variation can be expressed through the Van Deemter equation (eq. 1.4).
Equation 1.4: Van Deemter equation.

\[ H = A d_p + B \frac{D_m}{u} + C \frac{d_p^2}{D_m} u \]

where \( A, B \) and \( C \) are the constants that contribute to peak band broadening, \( d_p \) is the particle size of the packing material, \( D_m \) the molecular diffusion coefficient and \( u \) the linear velocity of the mobile phase. 

The equation can be graphically represented by a plot of \( H \) in function of \( u \) (fig. 1.1).

![Figure 1.1: theoretic curve of the Van Deemter equation](image)

The lower point of the curve represents the best working condition, since it is evident that before and after this point plate height increases.

The A term, or eddy diffusion, is independent on the mobile phase linear velocity and it is proportional to \( d_p \). The diffusion of the solute molecules occurs as a result of multiple flow paths present within the column bed. Since the length of the different paths is not homogeneous, molecules can travel different distance before reaching the detector (fig. 1.2).

![Figure 1.2: representation of the A term of the Van Deemter equation](image)

This term is lowered in columns packed with regularly shaped particles (spherical) as they form a more regular packed bed, while not modifying the curve shape (fig. 1.1) [1-3].

The B term is also known as the longitudinal diffusion term. It contributes to peak broadening only at flow rates below the minimum plate height (fig. 1.1). Molecular diffusion takes place independently on the longitudinal flow direction. The B term is the result of concentration differences in the mobile phase: in the center of the peak zone the concentration is at the maximum while before and after this region it decreases (fig. 1.3).

![Figure 1.3: representation of the B term of the Van Deemter equation](image)
This parameter is inversely proportional to \( u \) (eq. 1.3), meaning that the faster an analyte zone passes through the column, the less band broadening will occur. The B term is affected by viscosity, temperature and molecular weight, since it is directly proportional to \( D_m \), [1-3].

Finally the C term, or the resistance to mass transfer, relates to the mass transfer of the molecules between the stationary (\( C_s \) term) and the mobile phase (\( C_m \) term) (fig. 1.4).

![Figure 1.4: representation of the C term of the Van Deemter equation [3].](image)

The first contribution is determined by the amount of the stationary phase, the distance that sample has to travel and the extent of interaction of stationary and mobile phase, while the latter depends on the different velocities of the mobile phase into the column, lower close to the wall and faster in the center. The \( C_m \) term is positively affected by smaller diameter particles and lower diffusion coefficient (eq. 1.5).

**Equation 1.5: dependence of the C term on particle diameter.**

\[
C = f \left( \frac{d_p^2}{D_m} \right)
\]

The contribution of the C term to the peak broadening increases at high flow rates (fig. 1.1), because the exchanges of the molecules between mobile and stationary phase must be fast enough to keep up, if the equilibrium between the phases is to be maintained [1-3].

According to the Van Deemter equation (eq. 1.4), a decrease in particle size leads to a higher efficiency. But, in fact, this is not the only gain, since it makes possible to work at flow rates higher than the optimum velocity without losing efficiency.

The advantages of using small particles was combined with limitations, which soon became apparent and one in particular, i.e. the dramatic increase in backpressure.

According to Darcy’s law (eq. 1.6), the increase in backpressure is not linear:

**Equation 1.6: Darcy’s law.**

\[
\Delta P = \frac{u \cdot L \cdot \eta \cdot \phi}{d_p^2}
\]

where \( L \) is the column length, \( \eta \) the mobile phase viscosity, and \( \Phi \) the flow resistance.

Equations 1.5 and 1.6 show that for reduction of 3-fold in particle diameter, backpressure is proportional to the cube of particle diameters and it is not compatible with conventional HPLC instruments. For an identical column length under optimal-flow rate, a 1.7 \( \mu \)m column will generate a 27-fold higher backpressure than a 5 \( \mu \)m column [1-3].
REFERENCES

2.1.2 Monolithic supports

Monolithic columns represent an innovative analytical approach to reach high efficiency in a short time. They were developed early 1990s and the first generation of commercial columns were marked by Merck (Darmstadt, Germany) in 2000 [1,2]. Their shape could best described as a coherent, rigid, single rod obtained by in situ copolymerization of silica or different organic polymers, as styrene/divinylbenzene with monovinyl/divinyl methacrylate [3].

The smaller size of the silica skeleton, presumably, leads to higher performance analysis increasing the flow rate. The contribution of the mobile phase mass transfer term seems to be greater, while the contribution of the stationary phase mass transfer term seems to be lower compared to packed columns with a comparable pressure drop [4].

Their main advantage consists in the possibility to work at high flow rates without generating an exceeding high backpressure, thus making it possible to work with conventional HPLC instruments [5] while keeping high efficiency and resolution.

These features result from their innovative structure, consisting of:

- **macropores**: large pores (ca. 2 µm), that afford low flow resistance. They form a flow-channel network outside the monolith skeleton that rapidly transport the solute molecules by convection down the column and in contact with the active surface producing the chromatographic separation. They can be associated to the inter-particle volume of a packed column. These pores determine the column permeability and the derived backpressure. Thanks to their dimensions, the generated backpressure is much lower than that generated by conventional columns packed with particles of equivalent diameter. Therefore these phases make it possible to work at a higher flow rate, up to 10 mL min⁻¹, even with conventional HPLC instruments. The interstitial volume of a conventional packed bed column is about 40% of the total volume, while monoliths can achieve an interstitial volume, i.e. the volume of the macropores, as high as 80% [3,5]. The total porosity of the monolithic column is the sum of two contributions: internal porosity, \( \varepsilon_i \), due to the mesopores, and external porosity, \( \varepsilon_e \), due to the through pores (fig. 2.1 A);
- **mesopores**: about 12 nm. The total surface area of these particles is about 300 m²/g and provides the solute retention (fig. 2.1 B).

Surface and retention factors (k value) are smaller than those of a packed-bed column, because of the smaller amount of silica in the column, due to the higher porosity [4].

![Figure 2.1: macropore (A) and mesopore (B) structures in a monolithic rod [3].](image)

The Van Deemter curves show how the efficiency reached by monolithic columns is higher compared to conventional 5 µm, but comparable to a 3.5 µm column. The minimum plate height is lower and the curve flatter in the C region (fig. 2.2 A), leading to the possibility of working at higher flow rates without affecting efficiency. Conversely, the backpressure generated with monolith columns is lower than that generated by a comparable 5 µm and 3.5 µm column (fig.
2.2 B). Some studies showed that the backpressure is comparable to that generated by a column, of identical dimension, packed with 11 µm particles with an efficiency as good as that of a column packed with 3-3.5 µm particles [3].

![Figure 2.2: the Van Deemter curves (A) and pressure plots (B) of monolithic and 5 µm fully packed column [3].](image)

In the case of packed columns, inter-particles volume, and subsequently the permeability, depends on the particle diameter and the smaller the particles, the lower the permeability. However, column efficiency also depends also on particle dimensions and increases when small particles are used. Therefore performance and permeability in packed columns cannot be controlled independently, as they are both linked to the particle diameter. On the contrary, a control can be done with monolithic column dimensions, from mesopore residence time (diffusion-limited mass transfer) and macropore permeability (transport dominated by convection) [10].

Thanks to their large macropores and their rigid structure, their lifetime is about 2-fold higher compared to that of conventional packed columns; in some cases they can even be used for direct analysis of “dirty samples” removing the sample preparation step [6]. However, monolithic columns have also some limits: the number of stationary phases is limited: C8, C18 and bare silica, although Tanaka et coll. used other chemistry and even HILIC columns in their works [7-9]. Monolithic columns are not compatible with mass spectrometric (MS) detectors because of their bleeding and high flow-rate induces a huge consumption of mobile phases. They have limited stability at extreme pH and high temperature and can give a peak tailing even for neutral compounds. These disadvantages decrease their application fields, especially in routine analysis. Finally, the bare silica rod must be covered by a PEEK shrink-wrap during preparation to avoid the “wall effect”, that develops when the monolith is pulled away from the column wall, forming voids through which the mobile phase and solutes can flow, causing loss of separation.

Despite their high permeability, these columns did not have the expected success. Minimum plate height was about 20 µm, therefore a 10 cm column provided about 5000 plates, which are fewer than those generated by the newest sub-2 µm and core-shell columns [10]. Nowadays, a second generation of monolithic columns (Chromolith HighResolution) has been marked by Merck. Guiochon and Gritti highlighted three main disadvantages in the first generation columns:

i) large size distribution, variable geometry and random distribution of the macropores,
ii) large domain size and
iii) radial heterogeneity morphology that generates a velocity difference between the wall and core region of about 4% and 1.5% [11].

In the new generation of columns, macropore size is decreased to 1.2 µm, while mesopore size is increased to 15 nm (fig. 2.3 A).
The above described modifications do not affect surface chemistry: total surface area and total volume pore and even porosities (meso- and macro- total) are very similar to those of the first generation, which means that there are no evident changes in retention properties between the two generation columns [11].

However, the reduction in domain size causes a decrease in the eddy diffusion parameter, leading to a much higher separation efficiency, even at the cost of a about 3 fold higher column backpressure (fig. 2.4 A) [13,14].

Tallarek and coworkers demonstrated that the structure of Chromolith High Resolution columns is more homogeneous than that of the first generation columns. Together with the smaller macropore size, this homogeneity leads once more to a decrease of the eddy diffusion term and a more symmetrical peak shape, which in turn gives a performance comparable to results obtained with sub-2 µm and core-shell columns [6,11].

The minimum plate height is reduced by 39% from the 1st to the 2nd generation of monolithic columns (fig. 2.4 B), that means an enhancement of efficiency. Since flow rate of the minimum plate height of the 2nd generation columns is increased about four times and the curve is flatter in the C region, it is possible to work at higher flow rates without compromising analytical efficiency.
REFERENCES

2.1.3 High Temperature Liquid Chromatography (HTLC)

In HPLC, when a new method is developed, the first parameters to be optimized are usually column chemistry and mobile phase conditions [1]. Subsequently, resolution is improved by choosing the correct pH or by modifying the percentage of organic mobile phase or time gradient. Until recently, temperature had not been considered a variable with a significant role in HPLC method development. Probably, this is due to the fact that small changes in mobile phase composition give evident and immediate effects on selectivity than small changes in temperature. Most analyses are performed at room or just above room temperature, between 25°C and 40°C, weakly thermostating the column to improve repeatability of retention times.

Temperature has important impact on some physical parameters, such as viscosity, mobile phase polarity, surface tension and solute diffusivity. Thus by tuning temperature, it becomes possible to improve different analysis parameters and, as a consequence, separation too. Although the positive effects of high temperature or temperature gradient in LC have been discussed in the scientific literature for more than 30 years [2], only a small number of applications have been published and used routinely in analytical laboratories [3]. Mobile phase viscosity can be decreased by simply increasing the temperature. Thanks to the reduction in viscosity, efficiency is improved and pressure drop further decreased, making it possible to work at higher flow rates. Solute retention factor in RPLC is thermodynamically dependent on temperature, since it is a function of the free energy changes in the interaction between the solute and the stationary phase [4]. By substitution of enthalpy $\Delta H$ and entropy $\Delta S$ into the equation of free energy of solute transfer from mobile phase to stationary phase:

\[
\Delta G = -RT \cdot \ln \left( \frac{k}{\phi} \right)
\]

where $R$ is the gas constant, $T$ is the temperature in °Kelvin, $k$ is the retention factor and $\phi$ is the phase ratio of the stationary and the mobile phase, the van’t Hoff equation becomes:

\[
\ln k = \frac{\Delta H}{RT} + \frac{\Delta S}{R} + \ln \phi
\]

where $\Delta H$ and $\Delta S$ are respectively the enthalpy and the entropy of transfer of the solute from the mobile to the stationary phase. Temperature increase does not only produce a reduction in retention times, but can also induce changes in selectivity. This effect is especially noticeable for ionisable compounds since ionization equilibria are temperature dependent [3]. Temperature, as well as mobile phase pH, is a powerful tool to modify the dissociation rate in particular for basic compounds. According to some authors [5], if pH is varied of 2 units at a given temperature or if the temperature is increased of 60°C at a given pH the same dissociation percentage of basic compounds in acidic conditions is obtained. The use of temperature to modify selectivity may therefore be very useful, in particular for complex matrices with ionisable compounds. Peak symmetry of basic compounds is also improved, since the temperature accelerates the kinetic of the secondary interactions between solutes and stationary phase [6]. Better peak shape means also increase in resolution and peak height [6]. Another advantage due to the decrease of viscosity is a lower backpressure, that allows the use of higher flow rates even with conventional HPLC instruments (fig. 3.1).
As reported above, HT is particularly interesting when analyzing natural or very complex matrices. Thanks to its properties, it is allowed to increase efficiency by coupling several columns without exceeding backpressure limits. Very long columns at HT are able to reach efficiency values not achievable with columns of the same length at room temperature or even with new technology columns (i.e. sub-2 µm or core-shell column) alone [7,8].

About twenty years ago, Antia and Horvath found that temperature does not decrease the height equivalent to a theoretical plate (HEPT) at optimum velocity, but a change of the temperature from 25°C to 175°C leads to a 5- to 10-fold decrease in mobile phase viscosity, thus speeding up the interphase mass transfer. It is therefore possible to operate with much higher linear velocities keeping constant the theoretical plate number (N) at the optimal velocity [9].

Column efficiency is commonly described by the number of plate (N), that is related to HEPT (eq. 1.3) and which depends on the linear velocity of mobile phase, $u$, whose variation can be expressed through the Van Deemter equation (eq. 1.4) [4].

The effect of the temperature on the A term is not completely defined, although Carr found that elevated temperature will improve the laminar flow and mixing of analyte molecules from different flow channels, thanks to the increasing diffusivity. In any case, the improvement of what may not be highly significant [10,11]. The B and the C terms are both temperature dependent: the B term is directly proportional to the diffusion coefficient and increases with the increase of the temperature, while the C term is inversely proportional to the diffusion coefficient and temperature and it predominates at high flow rates.

Considering the Wilke-Chang equation (eq. 3.3), diffusion coefficients are directly proportional to the ratio $T/\eta$, for a given compound and mobile phase.

**Equation 3.3:**

$$D_m = 7.4 \cdot 10^{-8} \left(\frac{\Phi M}{T}\right)^{1/2} \frac{T}{\eta V_a^{0.6}}$$

where $D_m$ is the diffusion coefficient of the solute, $M$ the solvent molecular weight, $T$ the absolute temperature, $\eta$ the solvent viscosity at $T$, $V_a$ the molar volume of the solute at its boiling temperature and $\Phi$ the solvent association factor.

Equation 3.3 shows that by increasing the temperature, the diffusion of analytes in the mobile phase improves and this effect is enhanced, meaning that viscosity is inversely proportional to the temperature. Since linear velocity can be expressed as a function of diffusion coefficient ($D_m$) with:
Equation 3.4:
\[ u_{opt} = \frac{\nu}{dp} \cdot D_m \]

An increase in optimal velocity by increasing temperature is linked to the increase of \( T/\eta \). The Van Deemter curves in fig. 3.2, confirm that optimum velocities are shifted to higher values.

Figure 3.2: Van Deemter curves at different temperatures. Column: Zorbax Stable Bond 150 X 4.6 mm; 5 \( \mu \)m [Kindly given by dr. Davy Guillarme].

Vanhoenacker and Sandra also [3] demonstrated that the maximum plate number (N) that can be obtained with a given setup is relatively independent on the temperature, provided that the mobile phase is adequately preheated to prevent band broadening caused by temperature gradients between column and mobile phase. The minimum of \( H \) in Van Deemter curves shifts to higher values of \( u \) and the curve is flatter. The increase of the temperature, therefore, leads to an improvement in efficiency and in ratio of \( N \) and \( t_0 \) [10].

Thanks to the better mass transfer, the curve is flatter making it possible to work at higher flow rates, even beyond the optimal, without a significant loss in efficiency (fig. 3.2).

Another interesting benefit using HTLC, is the reduction of water polarity. Its dielectric constant can be reduced from 80 at 25°C to 35 at 200°C (fig. 3.3) [12]. Therefore water characteristics become closer to those of an organic solvent. Organic modifiers can be reduced or eliminated since water has a higher eluent power, similar to that of an organic mobile phase. In previous studies, a temperature increase of 3.75°C was found to have similar effect on retention as a 1% increase in methanol and a 5°C increase can be compared to a 1% change in ACN content [3].
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The reduction of viscosity has made possible to use 100% water as mobile phase, known as “Superheated Water Chromatography” [9,13], as well as unusual and more viscous solvents such as ethanol [3].

The use of pure water or other solvents has certainly some advantages:
- reduced or null toxicity of the mobile phase;
- reduced costs for the laboratory;
- possible hyphenation with very sensitive and universal detectors, such as flame ionization detection (FID);
- UV profiles could even be recorded at very short wavelength (190 nm), and thus many species not absorbing at conventional wavelength can be easily detected, although it can only be applied to simple mixtures, because interferences will occur with complex samples.

On the opposite some disadvantages have also been recorded:
- eluent strength is fixed, with the exception of using a gradient temperature, that however is unable to reach very high strength. Pure water at 150°C has an eluent power comparable to that of 50/50 (v/v) of water/methanol mixture at room temperature [5];
- hot water is a very aggressive solvent. Silica based columns are expected to rapidly degrade and the number of stationary phases suitable for superheated water is very limited;
- MS spectrometry-ESI mode can be less sensitive, because of the low percentage of organic solvent;
- sample solubility can be modified.

Although high temperatures offer the above advantages, the technique is seldom used in routine analysis, because of the problems it may cause:
- analyte stability: some compounds may degrade more quickly at high temperature than at room temperature. However, many compounds that are considered thermally instable (i.e. carbamates and phenylurea pesticides) are proved to be stable at high temperature [14]. From a theoretical approach, Antia and Horvath [5] predicted that on-column reactions would be irrelevant if the increase in the reaction rate caused by the high temperature was compensated by the decrease of analysis time, due to the increase of the mobile flow rate. The Damköhler number (Da) measures it, defined as the ratio between the column residence-time of the solute and the time needed for the reaction [9]. Fast analysis gives low values, as a consequence no on-column reactions can be expected. Solute degradation is not only due to temperature and residence time inside columns, but also to column nature and packing material, presence of metals [14], catalyzing siloxane bonds.

![Figure 3.3: decrease of water polarity with temperature increase [13]](image)
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[15], mobile phase composition, buffer system and pH [5]. Neutral pH conditions are generally to be preferred for HTLC analysis. In case of uncertain stability, it is therefore advisable to test it before to optimize or even validate a new analytical method.

In addition to sample degradation, column degradation can also occur. Conventional silica columns are stable in a pH range between 2 and 8, below this value the bonded phase is susceptible to hydrolysis, while beyond it, particle erosion can happen due to the silica matrix dissolution [14]. Moreover, high temperature can accelerate these phenomena. Progresses have been achieved to increase stationary phase stability at extreme pH, but fewer solutions have been found to withstand high temperature, even if hybrid silica-based stationary phases developed to resist at extreme pH normally are also stable at temperatures up to 100-120°C at neutral pH [16]. For example, organic/inorganic hybrid phases based on ethyl-bridge silanes, such as Acquity UPLC BEH Column (Waters Corporation), are stable in a range of pH between 2 and 12 and temperatures up to 90°C (fig. 3.4 A), while a sterically hindered phases, such as Stable Bond Column (Agilent Technology), provide protection from silica matrix dissolution at low pH and temperature up to 90°C (fig. 3.4 B).

However, column manufacturers discourage to work at both extreme pH and high temperature to avoid reduction in column life. New alternative stationary phases have been developed to overcome the problem of silica columns to be stable for long time at high temperatures, including metal oxide-based columns (e.g. zirconium, titanium, aluminium), porous graphitic carbon and organic polymers (e.g. polystyrene/divinylbenzene) [5,16,18]. Unfortunately, these stationary phases are often less efficient than the silica-based ones, especially with polar and basic compounds [5], and the separation processes, and therefore selectivity, are different [2]. It should be considered that not only the stationary phase is unstable at high temperature, but also the column hardware needs to be adapted, changing PEEK with stainless steel parts.

Good results in HTLC can only be obtained by a specially designed chromatographic system that minimizes both thermal mismatch and extra-column band broadening. Stable column temperature can be obtained by using different ovens: air-bath, block-heater or water-jacket oven [2].

In the past years, eluent preheating has been found necessary to avoid the formation of axial and radial temperature gradients inside the column. This is recognized as “thermal mismatch”, defined as the variance of temperature between the column and the mobile phase, that generates temperature differences inside the column [19]. Thermal mismatch between the incoming mobile phase and stationary phase leads to a severe band broadening or even peak splitting. It is not necessary to have a long preheating tube, that could produce peak distortion, but a tube of a
length suitable to keep the incoming mobile phase within a ±5°C range compared to the column is enough to maintain good performances [5]. Heat can also be generated inside the column, in consequence of the flow rate passing through the column. The viscous heat dissipation becomes a problem, in particular when the temperature is low and the pressure is high. However, thermal mismatch is less critical when working with both high temperature and pressure. Temperature can more easily be controlled with column diameters smaller compared to the conventional ones (e.g. 2.1 vs. 4.6 mm I.D.) thanks to the improved heat exchanges with the environment.

In addition to mobile phase preheating, mobile phase cooling is required when using an UV detector to avoid baseline noise and to preserve the UV cell. Moreover, if the analysis is carried out with superheated solvents, they could be present in vapour state, instead of liquid, therefore a small pressure (ca. 20 bar) has to be applied to maintain the liquid state after the UV detector.
REFERENCES

2.1.4 Ultra High Performance Liquid Chromatography - UHPLC

The reduction of particle diameter is one of the most interesting approaches both to increase efficiency and decrease analysis time. In the 1970s, 10 µm irregular particles were generally employed in HPLC, while in the 1980s their diameter was reduced to 5 µm and in 1990s down to 3.5 µm, at the same time particles became more and more spherical and regular. Currently, columns packed with particles with 2 µm range are widely available [1] and marketed by several brands with all chemistries. This new chromatographic approach shares the same rules and principles as conventional HPLC, but it is known as Ultra High Performance (or Pressure) Liquid Chromatography (UHPLC), as it can exploit sub-2 µm column power operating above 400 bar, the backpressure withstood by conventional systems.

Efficiency and analysis time can both be improved by using small particles. The reduction of particle diameter, down to sub-2 µm, compared to 5 µm columns enables us to:
- to speed up the analysis by a factor of 9, while keeping a similar efficiency,
- obtain a theoretical 9-fold increase in efficiency with equivalent analysis time [2].

Column efficiency can be expressed through the number of theoretical plates (N) or height equivalent to a theoretical plate (HEPT) in agreement with basic chromatographic principles and from van Deemter equation (eq. 1.4).

\[
N = \frac{1}{C \cdot \alpha^2}
\]

The A and the C term of van Deemter equation (eq. 1.4) present a particle size dependency [4]. The C term is the contribution to the plate height resulting from the resistance to mass transfer of the sample molecules in the stationary, mobile and stagnant phase. Since the particle volume where analytes can diffuse is smaller, the C term is reduced consequently, being it proportional to the square of the particle diameter (eq. 1.5). This means that smaller particles produce a reduction of the C term and thus the total plate height [5], with an additional reduction of the eddy term. The optimum linear velocity is therefore shifted to higher values when particle diameter decreases (fig. 4.1), thus making possible to work at flow rates higher than the optimal value without a significant loss of efficiency, since the mass transfer improves with 1.7 µm compared to 5 µm particles and the C term region of the van Deemter curve is flatter (fig. 4.1) [3,4].

Efficiency (N) is inversely proportional to the particle diameter (dₚ), as shown by eq. 4.1:
Equation 4.1: efficiency as function of particle diameter.

\[ N = \frac{L}{h \cdot d_p} \]

where \( L \) is the column length, \( h \) is the reduced plate height and \( d_p \) is the diameter of the particles. If the particle size is lowered by a factor of three keeping the same column length, for example from 5 \( \mu \)m to 1.7 \( \mu \)m, \( N \) will be three-times higher: this is an ideal condition for high efficiency analyses, while for high throughput analyses column length can be reduced three times without changes in performance [2,3]. Therefore, the same efficiency obtained with a conventional 150 mm column with 5 \( \mu \)m particle diameter can be attained with a column shortened by a factor equivalent to the particle diameter reduction, but in a shorter time (eq. 4.1) [3].

The advantage of using small particles soon displayed its limits and one in particular: the dramatic increase in backpressure. Moreover, while the diameter of particles decreased over time leading to higher analysis efficiencies, since 1970s there have been no improvements for instruments in backpressure stability and there were not adequate to the sub-2 \( \mu \)m separation potential. According to Darcy’s law (eq. 1.6), the increase in backpressure is not linear, but proportional to the cube of particle diameter at the optimal flow rate value and it becomes incompatible with conventional HPLC instruments [6]. A 1.7 \( \mu \)m column under optimal-flow rate generates a 27-fold higher backpressure than a 5 \( \mu \)m column for an identical column length. Newly developed instruments with a completely updated system design and advanced technology overcome this limit. The first system available on the market was the Waters ACQUITY UPLC System in 2004 [7,8], although nowadays several suppliers market instruments able to operate with very high pressure (i.e Shimadzu Corporation, Agilent Technologies, Thermo Fisher Scientific, etc.). All new instruments are designed for low systems and dwell volume to minimize the dispersion of the sample separation and take full advantage of the sub-2 \( \mu \)m technology. Extra-column band-broadening becomes a critical parameter because of the small dimensions of the sub-2 \( \mu \)m columns. In conventional HPLC, this parameter is not highly considered, as it only slightly affects the overall broadening of the peak, as it is smaller than the column volume. The ideal chromatographic system should have the ratio of extra-column variance (\( \sigma^2_{\text{ext}} \)) to the total variance (\( \sigma^2_{\text{tot}} \)) lower than 10% (fig. 4.2).

![Figure 4.2: band broadening due to extra-column variance (red line) and total variance (black line) [Kindly given by dr. Davy Guillarme].](image)

\[ \frac{\sigma^2_{\text{ext}}}{\sigma^2_{\text{tot}}} \leq 10\% \]

In conventional HPLC instruments, pumps withstand a maximum backpressure of 350–400 bar, depending on the instrument characteristics, while new designed UHPLC pumps could work under a backpressure up to 1300 bar [7]. Peaks are narrower than those obtained in conventional analysis, so even detectors have to be fastened to acquire peak signals correctly. In UV detectors, the sampling rate value must be increased enough to record enough data points across the peak for a correct quantification but not
to lose signal because of the increased signal-to-noise value. In addition, the detector cell should be small enough to avoid band broadening, but have a sufficient path length to maintain the required sensitivity. MS detection also improves with UHPLC systems since flow splitting is no longer needed, being the optimum flow rate for UHPLC columns compatible with MS systems. Sample concentration therefore increases at a higher source ionization efficiency and, as a consequence, so does sensitivity [9]. Even MS detector should be very fast: scan acquisition must reach at least 10000 uma/sec and dwell time reduced to < 5 ms for SIM and SRM modes [10].

Last, a sample injection in UHPLC instruments should be characterized by fast and pulse-free injection, low sample volumes, usually between 1 and 5% of column dead volume, and minimal carryover [8]. Columns also need to be modified to be stable at very high pressure. In 2000, a first generation of hybrid particles was developed by Waters. These columns present a methyl group that helps mechanically to enhance the strength in a wider range of pH, but they still do not withstand very high pressure (fig. 4.3 A). A second generation of hybrid particles enhanced their chemically stability which made it possible to use them under UHPLC conditions. The 1.7 µm Ethylene Bridged Hybrid (BEH) particles ensure the stability of the columns under extreme conditions of temperature and pH (fig. 4.3 B) [7,11].

![Figure 4.3: hybrid particles developed by Waters. A) first generation particles; B) second generation particles][12].

Other brands tried too to enhance column stability, for example, Agilent developed Stable Bond (SB) and Extra Density Bonded (XDB) technologies, consisting of nonfunctional silanes with bulky diisobutyl or diisopropyl side chain groups, that sterically protect the key siloxane bond to the silica surface [8].

Forcing liquid through column packed with small particles could generate heat (eq. 4.2), that can increase mobile phase temperature and generate an unwanted temperature gradient inside the column or even degrade sample.

**Equation 4.2: dependence of generate heat to flow rate and backpressure.**

\[
\text{power} = F \cdot \Delta P
\]

Generated heat can be dissipated both along and across the column, allowing the formation of axial and radial temperature gradients, respectively [17-19]. Usually when the radial temperature gradient is small, the longitudinal gradient is large and vice versa. This temperature gradient can affect both retention and efficiency and it depends on how column temperature is controlled. When it is kept constant, i.e., with forced-air ovens or water-baths, a radial temperature gradient can occur, meaning that column wall regions are colder than the central region (fig. 4.4).
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This temperature mismatch inside the column can cause loss of efficiency [19]. When the outlet is higher than the inlet temperature, due to a slow heat transfer (i.e. still-air oven), a longitudinal gradient temperature occurs. Unlike the radial temperature gradient, this phenomenon does not affect efficiency, but retention.

Gritti and coworkers studied the development of temperature inside columns trying to find an equation that correctly describe this phenomenon [14]. Fig. 4.5 clearly highlights the increase of temperature in both axial and longitudinal column dimensions. The behavior of columns of different lengths is different, although all the four columns present temperatures higher in the outlet region and wall temperature higher than that of the bed [14].

There are four different approaches to avoid these unwanted effects:

1. smaller diameter columns (i.e. 1 mm) can be used to reduce the flow rate and minimize the frictional heating [5,7,11,15];
2. several shorter columns can be coupled using an active cooling between the columns, but this solution cannot be used if the original separation is carried out on a 50 mm column length [15];
3. the column temperature can be lowered in method transferring to UHPLC [15];
4. method for UHPLC system can be redeveloped [15].

Another advantage underlying of the success of UHPLC technology is the easy transfer method from HPLC systems. UHPLC can theoretically give the same separation as HPLC provided with an identical stationary phase independently of its length and particle dimensions. In an isocratic mode [16], the injection volume and the mobile flow rate should be scaled down to be adapted to the new column dimensions and volumes, while keeping mobile phase composition and temperature constant. In the gradient mode, the method translation is not as simple and direct as in the isocratic mode. The injection volume and the mobile flow rate must be adapted with the
same rules as those used for the isocratic method transfer. Special attention should be paid to the dwell volume of the system, that is the volume related to the gradient delay to reach the column inlet (fig. 4.6), to avoid changes in the selectivity for the earlier peaks and in the gradient time. It is therefore necessary to keep constant the ratio of the system dwell time and column dead time.

Figure 4.6: volume delay [Kindly given by dr. Davy Guillarme].

The gradient profile between the two methods must be unvaried to keep the same separation [17]. For this reason the gradient volume has to be scaled in proportion to the number of column volumes passed through the column during the HPLC gradient to have an equivalent amount in the transferred UHPLC gradient. In gradient mode, several authors have also demonstrated that with the use of sub-2 \( \mu \)m packed columns, peak capacity can be improved working with flow rates even higher than the optimal one [2]. This is possible as column dead time \((t_0)\) has a stronger influence on this parameter than on efficiency \((N)\) (eq. 4.3).

Equation 4.3: peak capacity equation.

\[
PC = 1 + \frac{\sqrt{N}}{4} \cdot \frac{t_{\text{grad}}}{t_0 \cdot \left(1 + \frac{t_{\text{grad}}}{2.3 \cdot t_0 \cdot \Delta \Phi \cdot S}\right)}
\]

Free Excel spreadsheet that gives directly the new working conditions [18] can be used to speed up and facilitate the operations of the method transfer with different instruments and columns.
REFERENCES

2.1.5 Core-shell technology

Over the years, particles formed by solid core and covered with porous shell have been called in different ways: superficially porous, fused-core and core-shell particles. For the purpose of this chapter, the term “core-shell” has been chosen to describe these particles in all the steps of development.

The idea to use a thin film of porous stationary phase on a solid particle, in this case glass, was introduced by Horvárh and co-workers in 1960s [1], ten years later Kirkland, presented a 50 µm solid silica-based core covered by a silica porous shell. They were not immediately successful, mainly because of the strong development of the classical totally porous particles with a smaller diameter [2]. In the early 1990s, Kirkland rediscovered the advantages of core-shell particles and tried to report on their use and developed a 7 µm particle with a 1 µm shell thickness, for fast HPLC [3]. It was marked under the name PorosheII™ by Agilent and used especially for large biomolecule separation [4]. The most important evolution in this technology occurred in 2007, when the modern sub-3 µm core-shell particles become available once again thanks to Kirkland. These particles provided a much faster separation, compared to the fully porous ones.

Currently several manufacturing companies marked sub-3 µm and sub-2 µm core-shell columns [5]. Although columns from different brands might differ in terms of the diameter of the solid core or thickness of the porous layer, they all consist of a solid silica core covered by a thick layer of porous silica gel (fig. 5.1).

![Core-shell structure compared to a fully porous particle](image)
At present, the commercially available fused core columns are:

<table>
<thead>
<tr>
<th>Vendor</th>
<th>Column name</th>
<th>Particle diameter (µm)</th>
<th>Shell diameter (µm)</th>
<th>Stationary phase chemistry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agilent</td>
<td>Poroshell 300</td>
<td>5</td>
<td>0.25</td>
<td>C18, C8, C3</td>
</tr>
<tr>
<td></td>
<td>Poroshell 120</td>
<td>2.7</td>
<td>0.5</td>
<td>C18, C8, HILIC, RP-amide, phenylhexyl, pentafluorophenyl</td>
</tr>
<tr>
<td>Advanced Material Technology</td>
<td>Halo</td>
<td>2.7</td>
<td>0.5</td>
<td>C18, C8, HILIC, RP-amide, phenylhexyl, pentafluorophenyl</td>
</tr>
<tr>
<td></td>
<td>Halo Peptide-ES 160 Å</td>
<td>2.7</td>
<td>0.5</td>
<td>C18</td>
</tr>
<tr>
<td>Sigma-Aldrich</td>
<td>Ascentis Express</td>
<td>5</td>
<td>0.6</td>
<td>C18, C8 RP-amide, phenylhexyl, pentafluorophenyl</td>
</tr>
<tr>
<td></td>
<td>Ascentis Express</td>
<td>2.7</td>
<td>0.5</td>
<td>C18, C8, HILIC, RP-amide, phenylhexyl, pentafluorophenyl</td>
</tr>
<tr>
<td></td>
<td>Ascentis Express Peptide-ES 160 Å</td>
<td>2.7</td>
<td>0.5</td>
<td>C18</td>
</tr>
<tr>
<td>Phenomenex</td>
<td>Kinetex</td>
<td>5</td>
<td>nf</td>
<td>XB-C18, C18, phenylhexyl, pentafluorophenyl</td>
</tr>
<tr>
<td></td>
<td>Kinetex</td>
<td>2.6</td>
<td>0.35</td>
<td>XB-C18, C18, C8, HILIC, pentafluorophenyl</td>
</tr>
<tr>
<td></td>
<td>Kinetex</td>
<td>1.7</td>
<td>0.23</td>
<td>HILIC, pentafluorophenyl</td>
</tr>
<tr>
<td></td>
<td>Kinetex</td>
<td>1.3</td>
<td>nf</td>
<td>nf</td>
</tr>
<tr>
<td>Thermo Scientific</td>
<td>Accucore</td>
<td>4</td>
<td>nf</td>
<td>C18, aQ, RP-MS, HILIC, phenylhexyl, pentafluorophenyl</td>
</tr>
<tr>
<td>Macherey-Nagel</td>
<td>Nucleoshell</td>
<td>2.6</td>
<td>0.5</td>
<td>RP-18, HILIC</td>
</tr>
<tr>
<td>Sunniest</td>
<td>SunShell</td>
<td>2.6</td>
<td>0.5</td>
<td>C18</td>
</tr>
<tr>
<td>PerkinElmer</td>
<td>BrownLee SPP</td>
<td>2.7</td>
<td>0.5</td>
<td>C18, C8, HILIC, RP-amide, phenylhexyl, pentafluorophenyl, Peptide ES C18</td>
</tr>
<tr>
<td>Commercially non available</td>
<td>Eiroshell</td>
<td>1.7</td>
<td>0.35</td>
<td>C18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.7</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.7</td>
<td>0.15</td>
<td></td>
</tr>
</tbody>
</table>

Core-shell technology columns have quickly gained in popularity in analytical laboratories during last years because of their high performances. Their characteristics can be highlighted through the van Deemter curves (fig. 5.2).

A study by Cunliffe et co-workers in 2007, compared the performance of two core-shell columns (Halo and Express) and three sub-2 µm columns (Acquity, Zorbax and Thermo) [7].

Figure 5.2: Van Deemter curves for the 5 columns tested [7].
The van Deemter curves of these five columns clearly show how the efficiency of the two core-shells are only slightly lower than that of sub-2 µm columns. The core-shell columns can be used as an alternative to sub-2 µm ones with a loss of performance only of 20% [7].

![Figure 5.3: narrow distribution of the core-shell particles versus traditional porous ones](image)

In core-shell technology, the A term in the Van Deemter equation (eq. 1.4) tends to be lower. Its reduction is probably due to a better particle size distribution, compared to that of conventional fully porous columns. Core-shell particles, therefore, permit a more homogeneous packed bed, thanks to their more regular shape and size (fig. 5.3). The probability that solute molecules follow the same pathway, without slowing down into the column, is higher in core-shell. A better peak shape and increase in separation performance are the direct consequences of the smaller dispersion of the molecules into the stationary phase. Studies from different research groups [2,3,9-12] investigated the particle size distributions by comparing different core-shell and totally porous columns. Scanning electron microscopy (SEM) helped develop a real picture of the packed bed. All the authors agreed on the narrower and more uniform particle distribution in core-shell columns. Size distribution is very regular, with a relative standard deviation (RSD) of ± 5% [13].

![Figure 5.4: traditional chromatography versus core-shell technology van Deemter plot](image)

Gritti et al. also suggested that the roughness of the core-shell particles might lead to a more homogeneous packed bed, because rough surfaces produce a high friction between the particles, which decreases the amount of strain tacking place during the establishment of backed bed. [13-15]. Different studies reported a 40% reduction of the A term compared to values of totally porous particles for 4.6 mm I.D. columns (fig. 5.4) [10,16].

A reduction of the B term value between 30 and 50% for core-shells is expected compared to the value of fully porous particle columns [14]. This result could be explained by the presence of the solid core [9], that provides a lower volume accessible for the sample diffusion. However, the smaller B term has a relatively little impact on HEPT when the flow rate is at the optimum or slightly higher (ca. 10%) (fig. 5.4) [17]. Its benefit becomes relevant when columns operate at lower flow rate, for example when different columns are coupled together to reach a higher efficiency [10]. All core-shell columns have a similar B term value [3].

Smaller C term of Van Deemter equation makes it possible to apply flow rates beyond optimal value without compromising column performance. The C term is the key parameter for the efficiency loss when flow rates are increased beyond the optimal velocities to achieve faster analysis. In theory, the C term should decrease, when the thickness of the porous layer diminishes, because the distance along which the molecules have to diffuse is lower (fig. 5.5).
A comparison of the C term values of sub-2 µm and core-shell columns clearly shows the benefit of the solid core in the latter ones (fig. 5.5); a larger C term is observed in totally porous particle columns, even if their diameter is smaller than that of core-shell columns. In a column packed with the 1.7 µm totally porous material, an analyte can travel over a total distance of 1.7 µm in order to fully penetrate the particle and migrate out [3]. This distance is nearly twice compared to the thicker layer of the core-shell columns. The C term can differ between the superficially porous columns according to the manufacturers: thinner shells give lower C terms, and therefore the possibility to work at higher flow rate without compromising column performance [3]. Gritti et al. found that for all compounds with low diffusivities (i.e., peptides, proteins), the mass transfer kinetics are faster and the C term is about twice lower than with a column packed with totally porous particles [13], in the case of small molecules the C term is similar to that of totally porous particles [12]. This is unexpected since the morphology of core-shell particles was developed to reduce the intra-particle diffusion distance, decreasing the internal mass transfer resistance. Gritti et al. [2] studied this event in detail and concluded that for small molecules, the dominant contribution to the C term was the external film mass transfer, not the intra-particle mass transfer resistance. For larger molecules, the C term varied with core-shell columns, but it mostly depended on the pore size, that offer variable accessible volumes to the solutes. It can also be linked to different roughness of particle surface where the mass transfer through the outer stagnant mobile phase is reduced [18,19].

Analysts’ interest in core-shell columns is not only restricted to the comparable efficiency, but also include the lower backpressure generated compared to the sub-2 µm particles. Cunliffe et al. [7] studied backpressure of three sub-2 µm and two core-shell columns at different flow rates (fig. 5.6).
Acquity particles, i.e. those with the smallest diameter in this study, generated the highest backpressure, therefore presenting the lowest permeability, followed by the two other sub-2 µm columns (Thermo and Zorbax). Halo and Express columns give a backpressure that is 2-fold less than the other packed columns (fig. 5.6). This is due to the larger dimension of the particle diameter of core-shell columns that plays an important role in Darcy’s law (eq. 1.6). This feature has two important advantages:

i) it is possible to work with core-shell columns on conventional HPLC instruments reaching efficiencies only a little lower (20%) than sub-2 µm columns and

ii) flow rate higher than the optimal can be used without losing column performance because the C term in van Deemter equation is low.

It seems therefore possible to transfer method developed for UHPLC directly to a conventional HPLC by slightly affecting efficiency [5]. A study by Ruta and al. [5] showed a comparable selectivity between four core-shell and one fully porous sub-2 µm columns. However, also HPLC instruments need to be adapted: with core-shell columns, peaks become narrower, it is therefore mandatory to reduce extra-column volumes to avoid band broadening and use suitable parameters in data acquisition.

Several column chemistries are today available for core-shell columns, (C18, C8, C3, HILIC, silica, RP-amide, phenyl-hexyl, phenyl and pentafluorophenyl), but not in number comparable to that of sub-2 µm columns, meaning that not all separations can directly be transferred from sub-2 µm to core-shell columns [5]. The analyte retention is another important issue with core-shell particles: these columns might suffer of lower retention power in comparison with fully porous columns because of the silica solid core. On the opposite, the porous volume of core-shell particles is even higher compared to sub-2 µm particles (fig. 5.7).
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\[ V = \frac{4}{3} \pi \times R^3 \]

1.7 µm
2.6 µm (1.9+2*0.35 µm)
2.7 µm (1.7+2*0.5 µm)

Acquity
Vp=20.6 µm³
%porosity: 100%

Kinetex
Poroshell
Vp=28.7 µm³
Vp=44.9 µm³
%porosity: 64%
%porosity: 75%

Ascentis
Halo
Vp=20.6 µm³
Vp=61.8 µm³

Figure 5.7: porosity percentage of core-shell and sub-2 µm columns [5].

This aspect was confirmed by a study [5], where the retention factors of five columns (four core-shell and one sub-2 µm columns) were proved to be comparable. The small detected differences can be attributed to changes of carbon load, pore diameter, covered surfaces between columns together with the nature of end-capping and bonding phase process.

Sample loading capacity is another limit that was investigated, since it can modify the linear dynamic range of analyzed compound. This parameter is affected by different factors, including analyte properties, mobile phase conditions and surface chemistry of the particles [10]. Columns with high sample capacity usually provide a wider linear range, even considering that the loading capacity of a packed column is related to the carbon loading percentage [20]. However, the core volume of the 2.7 µm core-shell, that does not provide any retention, can be estimated at ca. 25% of the volume of 2.7 µm particles. This suggests that the sample loading capacity for the core-shell column should be approximately 25% lower than that of the sub-2 µm particle column [20]. This problem is particularly evident for ionized acidic and basic compounds. Two different research groups very similarly concluded that acidic mobile phases are not recommended because they decreases efficiency of two-fold compared to ammonium formate and potassium phosphate buffers, due to the saturation of the stationary phase and the impossibility for molecules to interact with non-ionized silanols [5,21].
REFERENCES

2.1.6 Supercritical fluid chromatography (SFC)

A fluid is defined “supercritical”, when it exceeds both its critical temperature and critical pressure (fig. 6.1).

![Phase diagram of CO2](image)

Figure 6.1: Phase diagram of CO2 [1].

The first applications of SFC occurred in the 1960’s, but it was not immediately successful. During the same years, two other analytical techniques, liquid chromatography (LC) and gas chromatography (GC), showed a wider range of applications and much more effort was done to develop these techniques unlike SFC. During the 1980’s, wall-coated open tubular columns, similar to those currently used in GC, brought new curiosity for SFC. However soon limits of this technique in terms of application fields again rose. Ten years later, a renewed attention occurred, thanks to the adoption of packed columns, usually applied to LC analyses [2]. However, SFC triggered an interrupted interest over the years probably thanks to its lower consumption of organic solvents, compared to other analytical techniques, high separation efficiency and fast analyses [3]. Lesellier and co. workers [4] showed that backpressure obtained in SFC at 20°C was dramatically lower (fig. 2.2 A, green line) than that obtained in HPLC at the same temperature (blue line). HPLC backpressure largely exceeded SFC backpressure, even when increasing the temperature at 40°C (pink line), therefore decreasing mobile phase viscosity. This feature (low viscosity of supercritical fluids) leads to work at higher flow rates with supercritical fluids than with liquids. Interestingly, the SFC curve is not straight as the other ones, since the fluid density, which depends on pressure, increases with the flow rate. As a result, the fluid viscosity increases as the flow rate, and the pressure variation is not linear.

Height equivalent to a theoretical plate (HETP) of SFC and HPLC have been compared in fig. 2.2 B, through van Deemter curves. For both LC and SFC analyses, the same column, apparatus and similar eluotropic strength were used to have reliable comparisons. The main differences between the two different techniques are: SFC (red and green lines) shows lower H values than HPLC (blue and pink lines), which is why it is possible to work at higher flow rates in SFC than HPLC without losing too much efficiency. There are no evident differences between the two temperatures used in HPLC, while the addition of 5% of methanol (green line) to pure CO₂ (red line) leads to small increase in the C-term dominated region of SFC curves. Because of the important reduction in viscosity of supercritical fluid compared with the hydro-organic mobile phase in HPLC, the diffusion coefficients are improved, which leads to an increase in the mobile phase linear velocity ($u_{opt}$). The B term of the curves is higher in SFC, since the latter is proportional to the diffusion coefficient, therefore the curve is shifted to higher $u_{opt}$ values, while the C term is lower as it is proportional to the ratio of the square of the particle size and to
diffusion coefficient, which results in an improved mass transfer and a lower H increases with flow rate.

**Figure 5:** A) variation of the pressure drop vs. flow rate for HPLC (blue and pink lines) and SFC (green line); B) variation of HETP vs. mobile phase linear speed in HPLC (blue and pink lines) and SFC (red and green lines) [4].

Basically, the main difference between SFC and LC is that the mobile phase in SFC is a dense compressed fluid, which expands if external backpressure is removed [5], while in LC, the mobile phase is a liquid.

The instruments used with these two different techniques are quite similar, except for two components. There is an additional module on the SFC pump to cool down the CO2 delivery pump, allowing to keep CO2 in a liquid state. In addition, a backpressure regulator (BPR), has to be added after the column to maintain the pressure higher than the critical pressure of CO2 in the whole system.

The supercritical state cannot be considered as a separate state. In this region, fluids are characterized by a higher density than gases, together with a lower viscosity and higher diffusivity than liquids. These properties give supercritical fluid higher solvating power than gases, lower backpressure and improved diffusion compared to liquids [6]. Decrease of pressure below the critical point leads the fluid into the gaseous phase with a consequent reduction of its eluent strength, while a temperature decrease leads the fluid into the “subcritical state”, before passing to the liquid state. This region is not well defined and a continuum state of matter between the two regions. It is not possible to know exactly the state where the analyses are performed in SFC, using mixtures of CO2 and MeOH. It is however possible to exploit all advantages of supercritical state even more subcritical conditions, where only one of the two critical conditions is respected, as the fluid properties do not change drastically. It is better to work in the sub-, instead of supercritical region for different reasons:

i) when working very close to the critical point, small changes in pressure or temperature can strongly effect fluid properties and, therefore, repeatability,

ii) the fluid compressibility is also reduced ensuring more constant liquid properties along the whole column,

iii) baseline noise in UV detector is lower when fluid and cell detector have the same temperature and, since the temperature is not very high, it is possible to work with thermolabile compounds, and

iv) column life is significantly better [7].

Carbon dioxide (CO2) is the most widely used fluid in SFC, thanks to its favorable and mild critical temperature (31°C) and pressure (7.3 MPa). Moreover, CO2 is cheap, non-toxic, non-corrosive, non-reactant, non-flammable and present low response with most detection systems [8]. CO2 has always been considered as a nonpolar solvent, because of its low dielectric constant and zero dipole moment [9]. Pure CO2 leads to a solvent strength comparable to pentane, and it is therefore not suitable to separate polar compounds. [10] To extend the polarity range of CO2, organic modifiers can be added from very low (2-5%), up to very high concentration (60%) [11]. A significant number of studies report the dramatic retention modification when a small amount
of modifier is added (<5%) and the increase of liquid properties is not directly proportional to the increase of percentage of organic modifier. A wide range of organic solvents can be used, such as methanol, ethanol, isopropanol, butanol, acetonitrile, tetrahydrofuran, dimethylsulfoxide, etc. [8]. Modifiers can produce some specific interactions such as hydrogen bonds or dipole-dipole, as a consequence tuning selectivity [10]. When polar solvents are added to CO₂, the eluent strength of the mobile phase dramatically increases. This is not only caused by the increase of mobile phase density, but also by changes of its polarity and absorption of the polar modifier at the surface of the stationary phase, that deactivates residual silanols [8,12]. The molecules of polar solvents probably also cluster together and form micro-environments with more polar features than the rest of the mobile phase. For this reason, polar solutes interact mainly with these polar clusters, while non-polar solutes tend to interact with the non-polar portion in the mobile phase, leading to an increase of solubility for polar compounds without modification of retention of non-polar solutes [5].

In spite of its interesting features, acetonitrile is barely used, as it is aprotic (not well adapted to elute polar compounds from the SFC column) and is a less eco-friendly solvent, thus reducing the greenness of the technique [13]. Methanol is probably the most widely used polar modifier in SFC. This solvent is the gold standard for SFC operation since it is completely miscible with CO₂ and, thanks to its polarity, it extends the solubility domain of the mobile phase. Obviously, the addition of methanol, or other solvents, increases the critical values of pressure and temperature, so, that if modifier is above 10%, the supercritical state is not reached and the mobile phase pH tends to be more acidic (pH around 5), because of the formation of carbamic acid.

Even with the addition of organic modifiers, it is still possible that the most polar compounds are not eluted from the column or that the peak shapes of ionisable compounds is unacceptable. Therefore, it is necessary to add a third component to the mobile phase, namely the additive. Generally, it is a small compound possessing either acidic or basic properties depending to the nature of the analytes that need to be analysed. Acidic additives are often used for acidic solutes, while basic additives are dedicated for basic solutes to have the compounds mostly under their non-ionized form. Conversely to the modifier, the additive is added in very small amounts, <1% v/v, and it allows to improve peak shape and efficiency [8]. It is usually added directly to the modifier, as it is not soluble or miscible with pure CO₂. Many articles report that additives can act in different ways:

i) covering the active sites of the stationary phase,
ii) changing the polarity of the stationary phase,
iii) suppressing the ionization of the analytes,
iv) enhancing ion pair formation with the analytes and
v) increasing the polarity and solvating power of the mobile phase [9,14].

In literature, several additives have been used to enhance separation, such as trifluoroacetic acid, citric acid, formic acid for acidic compounds and isopropylamine, diethylamine, triethylamine, ethyldimethylamine for basic compounds [3,8], but most of them are not compatible with mass (MS) since there are either non-volatile or suppress ionization in ESI. More recent studies reported the use of ammonium hydroxide as a potential substitute for basic analytes, as it is fully compatible with MS and easy to remove at a preparative scale [8,15,16].

SFC and LC separations can be achieved using similar stationary phases. Usually, SFC is considered as a normal phase technique, but in reality, its range of possible applications is larger. Indeed, in LC normal and reversed phase domains do not overlap, because of the presence of water. So, different columns and mobile phases must be considered in these two modes of chromatography (i.e. NPLC and RPLC) (fig. 6.3 A). In SFC, the situation looks different as analyses can be run with the same mobile phases but using polar (bare silica, diol, ethlypyridine, etc.) or non-polar stationary phases (C18, C8, phenyl, phenyl-hexyl, etc.) (fig. 6.3 B) [10]. This is an important advantage of SFC over LC, as it leads to a larger choice of column chemistries. In addition, it is also possible to attain some interesting selectivity and retention, simply by
connecting two stationary phases with an opposite interaction mechanism in series, while keeping the same mobile phase.

Another important advantage of SFC is the possibility of using a wider range of detectors, such as flame ionization (FID), UV, evaporative light scattering (ELSD) and MS detector [10,17]. Finally, SFC is exploited in several domains with more satisfying results than LC, such as chiral and preparative separations. For chiral analyses, it is indispensable to choose the right mobile phase since separation is based on interactions occurring between an analyte and the stationary phase and modifiers can modify both of them. For example, modifiers can alter the structure of the analytes or the sterical positioning of the chiral selector chains leading to mask or enhance particular interactions. Moreover, additives can enable or not elution of some compounds by ionization of their basic or acid functional groups, since only neutral compounds elute correctly from the column [2,6,18]. Thanks to its reduced viscosity and increased diffusivity, SFC is more productive than LC, making therefore easier to do large-scale separation. But, the most important advantage is that during isolation, CO₂ passes directly into the gaseous state, fractions are therefore more rapidly dried and the solvent waste reduced [18].

Recently, new instrument developments have afforded short analyses, making SFC performance comparable of those of UHPLC. Grand-Guillaume-Perrenoud et al. compared performance obtained with columns packed with sub-2 µm particles in UHPLC and UHSFC to evaluate both advantages and limitations of the two techniques. The Van Deemter curves and the pressure plots demonstrated that both optimal linear velocity and mass transfer were drastically enhanced for both chromatographic techniques as a consequence of particle size reduction. These observations were particularly unexpected in UHSFC, because of the reduction of mobile phase viscosity and the improvement of the diffusion coefficient compared to LC conditions. One of the main limits of new UHSFC instruments is the upper pressure limit, fixed at 400 bar ca., which is not sufficient when considering both the generated backpressure by sub-2 µm columns in presence of CO₂/MeOH mixture and the need to increase a pressure to maintain CO₂ in supercritical state. The method transfer between SFC and UHSFC could be an issue as both retention and selectivity...
can be modified by changing density with pressure, even if in this work this limit was observed only for a limited number of compounds [16].

REFERENCES

2.2 HPLC Techniques in Phytochemistry
2.2.1 Approaches for plant analysis

Plants produce a large amount of primary and secondary metabolites. The former are fundamental for plant life, as they are directly involved in normal growth, development and reproduction. The latter, that is secondary metabolites are not directly involved in growing processes, but they often play an important role in plant defence against predators, herbivore and microbial or fungal attack. Their absence does not result in immediate plant death, but it could lead either to problems for the organism’s survival, fecundity and aesthetics or cause no significant changes [1]. All these low molecular weight (i.e. < 1000g/mol) compounds constitute a very complex matrix, i.e. the metabolome. Although the metabolome’s size is not exactly known, scientists agree that it consists of more than thousands of different constituents [2-5]. This is probably due to the presence of several metabolic pathways developed by plants to survive in a range of environmental conditions.

Throughout the ages, secondary metabolites have found large use in human life as medicines, flavouring and even recreational drugs [1]. In particular natural products (NPs) have been essential sources for new drugs, since many of them have inspired synthetic molecules able to induce responses from human organisms [6]. This can be related to their chemical diversity or structural similarity of protein targets present in different species [7].

Content of active compounds in plants may vary greatly depending on both natural and man-made factors. Species and environmental factors, such as the area where they are cultivated, the climate (temperature, humidity, light, wind, etc.), the season and harvest time [8,9] account for the natural ones, while the different treatments of plants – drying, washing, crushing, pulverizing, storage or conservation for the latter.

As a result, quality control becomes essential to ensure not only product safety and reliability and repeatability of pharmacological activity, but also to understand their bioactivities and side effects of active compounds [10]. Several chromatographic techniques, such as high-performance liquid chromatography (HPLC), gas chromatography (GC), capillary electrophoresis (CE) or thin layer chromatography (TLC), can be applied to separate natural samples. HPLC has been recognized to be one of the most useful and versatile techniques in this respect since 1980 [7,8,11,12]. Unlike GC, it affords to operate with low temperatures, avoiding sample degradation, and the mixtures do not require derivatization to be made volatile. Different classes of compounds can be analysed by HPLC, mainly alkaloids, flavonoids, glucosinolates, isoprenes, polypropanoids, saponins, coumarines and carotenoids [12].

The large NP chemical variability makes it difficult to choose an appropriate detector to monitor different classes of compounds in a single analysis; moreover, the analytes in a sample can vary in concentration by as much as seven to nine orders of magnitude [4], which makes it mandatory to adopt an analytical system providing the highest resolution and sensitivity. In recent years, HPLC has significantly been developed, especially in terms of speed, chemistry of stationary phases, sensitivity and possibility to be hyphenated with different detectors [13]. Thanks to the larger choice of stationary phases, it is now possible to analyse almost all kinds of mixtures: from the most polar ones with HILIC columns to the most non-polar ones with cyano columns. Even pH is no longer an obstacle, as new manufacturing technology makes columns stable in a wide range (i.e. 2-12). The latest developments, sub-2 µm, monolith and core-shell columns, increase the performance in terms of resolution, speed and reproducibility [13,14]. Chromatographic systems can be coupled to detectors according to the purpose of the analyses and classes of compounds to be analysed. In general, they can be divided into two groups: detectors that simply record chromatographic trace, that are especially used for profiling and quantitation aims, such as UV, ELSD, and detectors able to generate a multidimensional data for online identification and dereplication, such as UV-DAD, MS and NMR [7].

Usually macroscopic and microscopic identifications or merely the presence of few markers are used to evaluate quality and authenticity of herbal medicines, to identify a herb or even to
quantify the herbal composition in an herbal product [10], even if they do not always correspond to the pharmacologically active compound(s). This determination does not permit a really complete description of the sample, because multiple constituents are synergically involved in the response. Therefore, it is not possible to break up the natural extract in different compounds, but the full extract can be analysed as a single “active compound”. The concept of phytoequivalence was developed in Germany to ensure the safety of herbal products by comparing sample profile(s) to the acquired profile of a clinically tested reference product [9,10]. Metabolomics have been defined as the technology designed to give the widest and essentially non-targeted information about the diverse population of small molecules present in living being [14]. A metabolomic analysis is a multi-step process consisting of sample collection, preparation, separation, detection and data processing [3]. Its primary aim is the non-targeted identification of all compounds in the sample [5]. Actually, different approaches have been adopted in function of the objective of the study: metabolic fingerprinting, metabolic profiling, dereplication or targeted analysis [2,4,5]. Metabolic fingerprinting aim is to create discriminating analysis able to compare pattern of different samples, without identifying each single compound presents in the mixture. Usually, extracts with comparable characteristics also present a comparable chromatographic fingerprinting. Thanks to this process, it becomes possible to determine the identity, authenticity and homogeneity of each sample and product lot [11]. All steps, especially separation, are as fast as possible to obtain a high throughput qualitative screening of the sample. In this case, resolution is not the major objective, on the opposite, it is important to reduce run time to few minutes by using short chromatographic columns and high flow rates [2,4,15]. Chromatographic fingerprinting has recently acquired a special interest among all quality control methods for plants and herbal products. It is nowadays accepted to evaluate product safety by WHO (World Health Organization), FDA (Food and Drug Administration), EMEA (European Medicine Agency), the Committee on Quality of Herbal Medicinal Products, the Committee for Proprietary Medicinal Products, the European Agency for the Evaluation of Medicinal Products, the Centre for Drug Evaluation and the Research, German Commission E, the British Herbal Medicine Association, the Indian Drug Manufactures’ Association and other official and nonofficial organizations [8,11,16].

Metabolic profiling leads to identify and qualify a limited number of metabolites present in the sample. They are chosen on the basis of a specific metabolic pathway, class or discriminating compounds, or biological activity. Dereplication is the process that permits to identify which natural samples contain new compounds of interest unlike those containing already known compounds [17]. In these analyses, it is particularly important to have a very high resolution so as to separate closely related compounds present in the sample, such as isomers or homologues, and obtain high-quality spectra without interferences for database analysis or spectral interpretation. This is an important tool for drug discovery, since it offers to obtain early structural information without having to resort to isolation, that is time-consuming, to development of bioactive-guided isolation procedure [2]. In this case, run time is sacrificed on behalf of efficiency. Column length is increased and particle size decreased, conditioned by backpressure limits [15], to achieve peak capacities as high as possible. The choice of the detector is fundamental and depends on the aim(s) of the study. The hyphenation of chromatographic techniques to detectors provides an efficient separation of metabolites together with other additional information: exact mass, UV profile or fragmentation pattern, etc. Several detectors are currently used for profiling and quantitation, namely UV, chemiluminescence (CL), refractive index detection (RID), evaporative light-scattering detection (ELSD), charged aerosol detection (CAD) and mass spectrometry (MS) [7], even if the hyphenation with an LC instrument leads to a simple and insufficient structural information for online identification and dereplication purposes. Other more powerful detectors are recommended for online structural detection, such as UV-DAD, MS, MS-MS, MS^n, time of flight (TOF), q-TOF-MS, Fourier transform (FT-MS) and NMR [7]. However, even with these detectors, dereplication and identification still remain a big
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challenge since any NP spectral databases are available. Moreover, unlike GC-MS, acquired data are instrument dependant and new structures must be studied *ex novo*. Since each detector has limitations and each hyphenated instrument should give the same clean response as that acquired from a pure product, it is possible to couple online more than one detector to have more information in a single run (i.e. HPLC-DAD-MS). If the sum of all the acquired data does not find a correspondence in NP database, more detailed information can be obtained with NMR, eventually hyphenated with an LC instrument [7].

LC-DAD is the simplest and the most widespread technique [10], that affords to record UV spectra of compounds with a chromophore, check for peak purity and compare the acquired UV spectra of the sample components to those of standards analysed with the same analytical conditions. This combination affords to follow not just one wavelength, but a set of UV wavelengths to monitor different classes of compounds in the same analysis. DAD enables us to identify compound families, but as such it does not provide with sufficient information to identify single compounds [7].

LC-MS is the key technique to identify compounds online. During past years, mass spectrometry has gained particular interest, because of its sensitivity, speed, broad application and does not require chromophores groups in the molecule [4], even if each of instrument set up presents peculiar characteristics. Several types of mass analysers giving different levels of information are available: MS, MS-MS, MS^n, TOF-MS, q-TOF-MS and FT-MS. They can produce nominal mass, empirical formula or diagnostic fragments. For dereplication purpose, molecular weight is fundamental, even when obtained with a single quadrupole, but it is mandatory to compare it with other MS data reported in literature obtained under different conditions so as to differentiate each molecule from possible adducts and fragments. However, this technique is not sufficient in itself to identify new compounds, but it can confirm or not the presence of a given compound.

High resolution mass spectrometry instruments, such as TOF-MS and FT-MS are suitable for both targeted and untargeted analyses. They are able to determine the exact molecular weight with an error of less than 5 ppm, even with unlimited number of compounds in a single run. With the exact mass, it is possible to go back to the molecular formula. This precision is essential to distinguish real molecules present in the sample from false-positive isobaric interferences. It is not possible to attribute the high efficiency to the TOF/MS alone, but it is essential to combine it with a powerful chromatographic system, such as UHPLC, to be able to distinguish isobaric compounds thanks to LC separation. Its main limits are: the elevated costs and the lower sensitivity compared to MS-MS and MS^n instruments. An effective solution is the hybrid q-TOF-MS that combines the high mass resolution and accuracy of TOF analyzer with the structural information, given by the fragmentation pattern obtained in a quadrupole [3,13].

Other information can be acquired by a collision-induced dissociation in MS-MS and MS^n. They are especially used in quantitative analysis, thanks to their high sensitivity. Multiple Reaction Monitoring (MRM) makes it possible to follow a limited number of specific MS-MS transitions. It is a very sensitive method as it can filter just the selected ions in the first quadrupole and in the third quadrupole one or two fragments obtained from the parent ion fragmented in the second quadrupole. Ion trap analyzers can also give an idea about molecular identity, thanks to the progressive fragmentation pattern of the structure. For example, the sugar sequence in glycosides can be highlighted, or the fragmentation of flavonoids can give the substituent position on the A or B ring, etc. But for analysis of totally unknown compounds is not sufficient [3,13].

In conclusion, there is no one best detector in absolute sense, but for the purpose of the research under investigation, some detectors can be more highly recommended because of the amount of information they can generated compared to others.
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3. Experimental part
3.1 Method Development
The study in this section describes simple and general rules to set up an analytical method in LC. Plant samples require to have high resolution analyses to allow the separation of the highest number of compounds. As plant extracts may consist of several classes of analytes with different polarity, work has to be performed under gradient conditions to permit the correct elution from the column of all them.

It is then possible to vary different parameters, such as:
- column chemistry,
- type of organic solvent,
- pH of the mobile phase and
- temperature.

Not all of the above generate the same results, for example temperature is useful to reduce analysis time, but it has lower impact on selectivity than small changes of pH or percentage of organic phase.

However most of the parameters present limitations: changes of selectivity by modifying the pH are only possible with ionisable molecules, temperature tuning requires adapted instruments and columns, some organic modifiers increase too much backpressure for conventional HPLC instrument and it is not always possible to have different column chemistries to test. It is therefore necessary to have an idea of the characteristics of the nature of the molecules present in the sample and use the most general conditions to start, such as a long C18 column, long and wide gradient, acetonitrile as organic mobile phase and a temperature of 40°C. Only after a first idea of the complexity of the mix, it is possible to start to tune each parameter and highlight possible advantages or disadvantages.

In this section, four different column chemistries, namely BEH C18, BEH C8, BEH Shield RP18 and BEH Phenyl, two types of organic solvent, acetonitrile and methanol, two pH, 2.2 and 5.5, and three temperature, 30, 45 and 60°C, were tested to find the best possible operative conditions.
3.1.1 General guidelines for method development of plant natural extracts to increase both efficiency and decrease time analysis *

*to be submitted to an international journal

ABSTRACT

Plant natural extracts are very complex mixtures that can contain a large number of compounds belonging to different chemical classes. This article reports simple guidelines to follow to approach to an unknown sample for the first time and to develop an effective analysis method rapidly. Different parameters must be tuned, such as organic solvents, mobile phase pH, temperature and column chemistry. Their impact on separation or selectivity can be different, but their simultaneous optimization can provide an optimized analysis method. UV is the most used detector for liquid chromatography (LC) instruments, although, often it is not sufficient to detect co-elutions requiring a more diagnostic detector (e.g. tandem mass spectrometry (MS/MS)) to increase selectivity. Complex matrices, such as natural samples, can cause other problems beside selectivity, making a big issue the quantification of the selected markers even on MS/MS detectors.

KEYWORDS: method development, HPLC, column chemistry, UHPLC, MS-MS.

1. INTRODUCTION

Method development is probably one of the limiting step in analysis of complex mixtures, in particular for plant natural extracts, since, raw extracts can contain a very high number of compounds belonging to different chemical classes. Liquid chromatography (LC) is the most popular and versatile technique to analyse samples containing low volatility components of plant origin [1-4].

In LC, several parameters concerning stationary and mobile phases have to be tuned to obtain the optimal analytical conditions to separate samples. The recent trend in chromatography is to speed up analyses, because of the increasing request of analyses, although complex matrices require a constant increase of efficiency. The introduction of ultra-high performance liquid chromatography (UHPLC) allowed to reach both these goals, using all the rules usually applied in conventional LC.

Monolithic, sub-2 μm and core-shell columns derive from relatively new technologies. Monolithic column shape can be considered as a coherent, rigid, single rod obtained by co-polymerization in situ of silica or different organic polymers [5]. They have an innovative structure consisting of macropores and mesopores [5-7], affording to use conventional HPLC instruments, because of low backpressure they produce at high flow rates [6,7].

The reduction of particle size is one of the most interesting approaches to increase efficiency and at the same time to decrease analysis time. In fact, reduction of particle diameter to sub-2 μm, compared to 5 μm generally adopted, enables to speed up analyses by a factor of 9, while maintaining a similar efficiency, or to increase efficiency theoretically up to 3-fold while keeping the same analysis time [8]. These performances also required renewed instrumentation to withstand both the 27-fold higher backpressure generated by the new columns and to speed up detector response to enable to acquire sufficient points to describe correctly a peak [9,10].

Core-shell stationary phases are characterised by a solid silica core covered by a thick layer of porous silica gel. A study by Cunliffe et al. in 2007 compared the performance of core-shell and sub-2 μm columns [11]. The core-shell columns exhibited a loss in performance only of 20% compared to sub-2 μm columns [11], but with a backpressure two-fold lower than with the sub-2
µm columns, thanks to the bigger particles. Core-shell columns are therefore able to reach high efficiency, even working on conventional instruments.

Temperature has not been considered, until recently, a variable with an important role in HPLC method development, since small changes in mobile phase composition gave more evident changes in selectivity when compared to small changes in temperature [12]. In reality, temperature has important effects on some physical parameters, such as viscosity, mobile phase polarity, surface tension and solute diffusivity. Not all the columns can withstand high temperatures, so the maximum effects of high temperature are obtained with column stationary phases with new designs, such as hybrid-silica columns or oxide-base columns [13-15], and, especially, adapted instruments [16].

Validation is necessary when a method is developed to be used in routine. Method validation is the process used to confirm that the analytical procedure employed for a given analysis is suitable for its intended use. Different parameters must be taken in consideration, UPS published specific guidelines defining eight parameters for a correct validation. For quantification purposes, HPLC is usually hyphenated with UV detectors, but for complex matrices more sensitive detector, such as mass spectrometry, should be used to avoid peak misinterpretation. This study aims to set up simple guidelines to choose the best analytical conditions to approach the analysis of a new plant extract. Because of the recent general trends, new available approaches are here tested to speed up analyses and/or to increase efficiency while highlighting their limits and advantages. Finally, an attempt to quantify polyphenols in teas is achieved to evaluate detector power with very complex matrices too.

2. EXPERIMENTAL

2.1 Chemical and reagents
The standard catechin flavonoids (+)-catechin (99%, C), (−)-epicatechin (96%, EC), (−)-catechin gallate (100%, CG), (−)-epicatechin gallate (99.1%, ECG), (−)-gallocatechin gallate (99.9%, GCG), (−)-epigallocatechin gallate (95.1%, EGCG), (−)-epigallocatechin (98.2%, EGC) and also gallic acid (99%, AC) were all obtained from Sigma–Aldrich (Milan, Italy) and stored at −20°C in darkness. A stock solution of these eight compounds at a concentration of 0.5 mg/mL was initially prepared in pure methanol, and working standard solutions were obtained by appropriate dilution with pure water. Pyrethrum standardized extract Pestanal® was purchased from Sigma–Aldrich (Milan, Italy) and stock solution was prepared in pure methanol. For the UHPLC–UV experiments, acetonitrile and methanol were of HPLC gradient grade from Panreac Quimica (Barcelona, Spain), formic acid was obtained from SDS (Peypin, France), acetic acid glacial acetic acid from Biosolve (Valkenswaard, Netherlands) and ammonium hydroxide from Fluka (Sigma-Aldrich Steinheim, Germany). For the UHPLC–MS/MS experiments, formic acid and acetonitrile (ACN) were of ULC/MS grade and purchased from Biosolve (Valkenswaard, Netherlands). Finally, water was obtained from a Milli-Q Water Purification System from Millipore (Bedford, MA, USA).

2.2 Instrumentation

2.2.1 UHPLC–UV experiments
UHPLC–UV experiments were performed on a Waters Acquity UPLC system (Milford, MA, USA). This instrument included a binary pumping system with a maximum flow rate of 2 mL/min, an autosampler with an injection loop volume of 2 or 5 µL used under full loop conditions, a UV–vis programmable detector and a column manager that included a column oven. Data acquisition, data handling and instrument control were obtained with an Empower Software v2.0. The UV detector time constant and data sampling rate were adjusted between 25–
100 ms and 20–80 Hz, to obtain signals of highest quality even with ultra-fast separations. The detector wavelength was set at 230 and 265 nm, respectively for pyrethrum standardized extract and catechin flavonoids as the best compromise to reach maximum absorbance for the compounds of interest.

2.2.2 UHPLC–MS/MS experiments
UHPLC–MS/MS experiments were performed on the same Waters ACQUITY UPLC system hyphenated with a triple quadrupole (TQD) mass spectrometer from Waters with an upper mass limit of m/z 2000. The TQD operated at a single mass resolution of m/z 0.7 FWHM. The ESI\textsuperscript{®} ionization source was used in the ESI negative mode, and ionization parameters, cone voltages and collision energies were optimized by infusing each compound (1 µg/mL) in 50:50 ACN:water plus 0.1% formic acid at a flow rate of 600 µL/min. Optimal cone voltage and collision energies values are summarized in Table 1. The capillary voltage and the source extractor voltage were set at 3000 and 3 V, respectively. The source temperature was maintained at 140°C, the desolvatation gas temperature and flow at 400°C and 800 L/h, respectively, and the cone gas flow at 50 L/h. MS/MS detection was carried out in the SRM mode, transitions are reported in Table 1. The collision gas flow was set at 0.2 mL/min of argon, and the entrance and exit potentials were adjusted to 1 and 0.5 V, respectively. Data acquisition, data handling and instrument control were performed using the Masslynx v4.1 software.

2.3 Columns
Separations were carried out on several analytical columns: a Hypersil Gold C18 (50mm × 2.1mm I.D., 1.9µm) provided by Thermo Fisher Scientific (Runcorn, UK), an Acquity BEH C18 (50mm × 2.1mm I.D., 1.7µm), an Acquity BEH C8 (50mm × 2.1mm I.D., 1.7µm), an Acquity BEH Shield RP18 (50, 100 and 150mm × 2.1mm I.D., 1.7µm) and an Acquity BEH phenyl (50mm × 2.1mm I.D., 1.7µm), all provided by Waters.

3. RESULTS AND DISCUSSION
Different parameters affect separation, such as organic modifier, pH, temperature and column chemistry. Each of these parameters should be tuned to develop an analytical method to obtain the optimal separation conditions. For most samples, a systematic, trial-and-error approach based on three successive steps can be followed. First, mobile-phase strength (%B) is varied until the right retention range is achieved, usually, 1 ≤ k < 10. Second, different separation conditions are explored to obtain acceptable selectivity (values of α) and resolution. Changes in %B (e.g. ±10% B) and temperature (e.g. 30–50°C) should be explored to improve selectivity. If some peaks are still overlapped and poorly separated, other conditions can be varied to improve selectivity (as described later on). The third step is to vary column conditions: column length and chemistry, particle size, and/or flow rate.

3.1. Mobile phase
Organic modifier and pH can be varied to improve selectivity depending on the sample features. In RPLC, different organic modifiers can be used to elute the most non-polar compounds, the retention (k) of all compounds decreases with the increase of the volume-percent of organic solvent (%B). A mobile phase that provides smaller k values is considered a “stronger” eluent; on the opposite, water is a “weak” solvent. Values of k typically decrease by a factor of 2 to 3 with an increase of 10% B [17]. Organic modifiers must be completely miscible with water, the less viscous possible and as transparent as possible at the lowest wavelengths for UV detection. Several organic solvents can be used, but the most employed are acetonitrile (ACN) and methanol (MeOH). Tetrahydrofuran is rarely used because of its high UV cut-off and non-
compatibility with PEEK instrument parts. Although ACN is considered sufficiently toxic to be limited in its use, it is probably the favourite solvent in most laboratories. Because it produces the lowest backpressure, it allows therefore to work at higher flow rates and it has a higher eluent strength compared to methanol.

Ionized acids and bases present much more polar features than their neutral corresponding items, being therefore less retained. A change in mobile phase pH, inducing an increase of solute ionization, leads to a decrease in retention time; in reality, reducing sample ionization is advisable, since it results in stronger retentions, more symmetrical peaks and robust RPC methods [17].

A simple sample, i.e. pyrethrum extract, was chosen to highlight the influence of all the parameters in method development. This extract consists of natural organic esters deriving from Chrysanthemum cinerariaefolium. Six different compounds have been isolated and identified, i.e. Pyrethrin I and II, Cinerin I and II and Jasminol I and II. They have a potent insecticidal activity, being active on the nervous systems of all insects, but they are totally harmless for human beings and mammals. They are non-persistent, being biodegradable when exposed to light, oxygen and high temperature [18-20].

First, two different organic solvents were tested, ACN and MeOH. As reported in fig. 1A, ACN gave a base line separation of all of the six compounds in 11 min. The separation with MeOH under the same gradient, is not acceptable, since in the first 9 min any peaks elute and Jasminol II is out of the gradient range (fig. 1B). This is due to the lower elution strength of MeOH compared to ACN. Increasing the MeOH percentage by keeping the same gradient slope as the above method, a separation fully comparable to that with ACN is obtained (fig. 1C).

Any logical reasons therefore induce to prefer MeOH to ACN, in addition considering that MeOH develops higher pressure than ACN, namely 283 and 443 bar.

### 3.2 Mobile phase pH

Beside organic modifiers, pH has an important role both for selectivity and peak shape for molecules presenting ionisable functions. Solute retention changes with pH only when the pH of the mobile phase is within $\pm 1.5$ units of the pKa value of the solute. As a consequence, mobile phase pH affects separation selectivity only with values similar to pKa of the sample
constituents. These conditions also afford the use of very low buffer concentration (10-20 mM), because of the high buffer capacity of mobile phase. A pH of 2.2 is easily achievable by simply adding 0.1% formic acid (FA) to both mobile phases to avoid baseline movements along the gradient. For pH between 2 and 3, ammonium formate is the preferred buffer, while for pH between 4 and 6 ammonium acetate has to be used. However extreme pH must not be adopted to avoid damage to the stationary phase. Low pH, in fact, can cause detachment of bonded chains from silica and high pH can hydrolyze silica and presents some compatibility problems with mass spectrometric detectors. Usually, the nature of most compounds in plant extracts is acidic, making advisable to work with acidified mobile phases. 0.1% FA and 10 mM ammonium acetate are therefore added both to ACN and MeOH to evaluate possible variations in selectivity, peak shape and eventually in retention time.

Figure 2: comparison of pyrethrum extract analyses with different organic solvents and pH. Column: Halo C18 150 X 2.1 mm I.D., 2.7 µm, temperature 45°C, 0.4 mL/min. A) black line: 45-85% ACN in 12 min and red line: 45-85% ACN + 0.1% formic acid in 12 min. B) black line: 55-90% MeOH in 10.6 min and red line: 55-90% MeOH + 0.1% formic acid in 10.6 min.

As shown in fig. 2A and B, no modification occurred by changing pH. This is because the investigated molecules do not present ionisable group. It is therefore advisable to investigate molecule nature before this step to avoid useless steps. In any case the simplest modifier has to be used because: i) less impurities are added to the mobile phase, ii) pH is subjected to fewer variations, and iii) variations due to the operators are limited.

3.3 Temperature

Only recently, temperature gained an important role in method development and routine analyses. Its lower consideration was because changes in solvent strength or percentage are more effective than temperature variations. This parameter is particularly interesting for the analysis of compounds that have no ionisable groups, therefore not sensitive to pH variations. Temperature has important impact on many physical parameters, such as viscosity, mobile phase polarity, surface tension and solute diffusivity. Mobile phase viscosity can be decreased by simply increasing temperature. Thanks to the reduced viscosity, efficiency is improved and pressure drop lowered, making it possible to apply higher flow rates or to couple two or more columns to increase efficiency [21]. Water polarity decreases while increasing temperature, therefore water characteristics become similar to those of organic solvents [22]. As a consequence, organic solvents can partially or totally be replaced by water or use less toxic solvents, such as ethanol.
General guidelines for method development of plant natural extracts to increase both efficiency and decrease time analysis

[22-24]. Last but not least, Van Deemter curves are flatter in the C region, thanks to the improved mass transfer, leading to work with higher flow rates without losing separation. Three different temperatures were evaluated: 30, 45 and 60°C.

![Figure 3: comparison of pyrethrum extract analyses with different temperatures. Column: Halo C18 150 X 2.1 mm I.D., 2.7 µm, 0.4 mL/min, gradient 45-85% ACN in 12 min. Blue line: 30°C, red line: 45°C and black line: 60°C.](image)

Analyses reported in fig. 3 clearly highlight the influence of temperature on retention time: increasing the temperature of 30°C, retention time of Jasmolin II decreases of about 15% (tab. 1), while increasing it of 15°C, retention time decreased of about 10%. Backpressure too is strongly influenced by temperature: at 45°C the pressure decreased of 20% compared to the analysis carried out at 30°C, while at 60°C the decrease was of 35% ca.

**Table 1: comparison of retention time and backpressure of the three temperatures.**

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Tr Cin II (min)</th>
<th>Tr Jas I (min)</th>
<th>Backpressure (bar)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30°C</td>
<td>6.40</td>
<td>11.05</td>
<td>352</td>
</tr>
<tr>
<td>45°C</td>
<td>6.00</td>
<td>10.33</td>
<td>283</td>
</tr>
<tr>
<td>60°C</td>
<td>5.50</td>
<td>9.55</td>
<td>234</td>
</tr>
</tbody>
</table>

This temperature (60°C) is the maximum temperature guaranteed by manufactures for core-shell columns. 45°C is therefore a temperature affording simultaneously reduction of analysis time and backpressure; in addition, column is less stressed and therefore its “life” longer.

### 3.4 Column chemistry

Silica is the most commonly used support for the production of LC columns. The high mechanical strength of its particles is a strong advantage affording the formation of packed beds stable for long periods and high operative pressures. Moreover, silica can be bonded to different ligands, such as C18, C8, cyano, phenyl, etc., useful to improve separation and modify selectivity. Silica begins to dissolve in the mobile phase at pH > 8, but bonded-phases can extend the range between 2 and 12. RPC packing usually are made by covalently reacting an organosilane with the silanols on the surface of a silica. There are eight different mechanisms that can affect column selectivity: (a) hydrophobic interaction, (b) steric exclusion of larger solute molecules from the stationary phase, (c) hydrogen bonding of an acceptor (basic) solute group by a donor (acidic) group within the stationary phase, (d) hydrogen bonding of a donor (acidic) solute group by an acceptor (basic) group within the stationary, (e) cation-exchange or electrostatic interaction between a cationic solute and an ionized silanol (–SiO−) within the stationary phase; also repulsion of an ionized acid (e.g., R–COO–), (f) dipole–dipole interaction
between a dipolar solute group and a dipolar group in the stationary phase (g) $\pi - \pi$ interaction between an aromatic solute and either a phenyl group (phenyl column), or a nitrile group (cyano column), (h) complexation between a chelating solute and metal contaminants on the particle surface [17].

For these reasons, different Hybrid Acquity BEH columns were tested, namely C18, C8, RP18 Shield and Phenyl. Methods were adapted to obtain the best separation for each column. The corresponding chromatograms are reported in fig 4. There are not so many differences between C18 and C8 columns (fig. 4A and B), since they share the same separation mechanisms. Acquity BEH RP18 Shield is characterized by the inclusion of a carbammate group in the side chain. This chemistry offers a higher selectivity than C18 and C8, especially for compounds with very close structures. In particular, the separation (fig. 4C) improves, between Cinerin II and Pyrethrin II, with a shorter analysis time. Finally, a phenyl column was tested. Fig. 4D shows the perfect baseline separation of all six compounds. This column is recommended, because it exploits $\pi - \pi$ interactions between the phenyls of the stationary phase and conjugated bonds present on the molecules.

![Figure 4: Comparison of pyrethrum extract analyses with different sub-2µm column chemistries. A) Acquity BEH C18 50mm × 2.1mm I.D., 1.7µm column, B) Acquity BEH C8 50mm × 2.1mm I.D., 1.7µm column, C) Acquity BEH Shield RP18 50mm × 2.1mm I.D., 1.7µm column and D) Acquity BEH phenyl 50mm × 2.1mm I.D., 1.7µm column.](image)

The importance of column chemistry is even better highlighted with the separation of seven different catechins and gallic acid on four columns: conventional C18 support, Hybrid BEH C18 column, Hybrid BEH RP18 support with a polar embedded group, and Hybrid BEH phenyl group support. Fig. 5 shows the chromatograms obtained with the four stationary phases. Fig. 5A and B present very similar chromatograms, as the two columns (both C18) share the same separation mechanisms. The separation is rather poor because CG and ECG and, in particular, EC and EGCG, are not baseline resolved. Phenyl support is not suitable at all, since CG and ECG co-elute and EC, GCG and EGCG are difficult to separate. Finally, fig. 5D shows a full baseline separation achieved with a RP18 Shield column. This column is particularly useful to separate very closely eluting peaks, thanks to its polar embedded group.
Figure 5: Optimal UHPLC chromatograms of an eight standard polyphenols mixture at 20 μg/mL obtained with various RP columns packed with sub-2 μm particles at 30°C. Numbers correspond to Fig. 1. \( F = 500 \) μL/min and a gradient procedure was used with \( A \) as pure water and \( B \) as ACN. 0.1% formic acid was present in both solvents. (A) Acquity BEH C18 50 mm × 2.1 mm I.D., 1.7 μm column, gradient 11–16.6%B in 3.52 min. (B) Hypersil GOLD C18 50 mm × 2.1 mm I.D., 1.9 μm column, gradient 10–16.3%B in 3.52 min. (C) Acquity BEH phenyl 50 mm × 2.1 mm I.D., 1.7 μm column, gradient 16.8–23.4%B in 0.6 min. (D) Acquity BEH Shield RP18 50 mm × 2.1 mm I.D., 1.7 μm column, gradient 13.3–22.2%B in 3.46 min. 1: C, 2: EC, 3: AC, 4: CG, 5: ECG, 6: EGC, 7: GCG, 8: EGCG.

3.5 MS/MS quantification

UV is the most used and simple detector hyphenated with LC instrument. It is highly sensitive, linear, versatile and reliable. All of the natural compounds are detected in the range of 200-550 nm. It presents however some important limits: it is not an universal detector, since compounds without chromophores cannot be detected [1] making it difficult to identify possible co-elutions and a sample component is detected, only if it absorbs at the chosen wavelengths or within the selected range. The use of mass spectrometric (MS) detectors are ever increasing not only for identification but also for quantitation purposes. The most important feature is that selectivity is dramatically increased. Different MS ionizations and analyzers are used for online identifications, but a quadrupole MS/MS analyzer is in general necessary for quantitation, in this case being mandatory a high selectivity to avoid analyte overestimation. MS/MS techniques make it possible by selecting univocally parent ions from the first quadrupole and the fragments formed in the second. Thanks to the HPLC separation, it is possible to locate precisely the pick of interest.

The mix of seven catechins and gallic acid was used to evaluate the selectivity of MS/MS detector. MS detector alone was not able to discriminate the eight compounds, since the mixture contains different couples of isobaric compounds. The hyphenation with an instrument that provides separation, such as UHPLC, is therefore necessary. The optimized method reported in § 3.4 was applied with MS parameters and conditions (tab. 2) chosen by infusion of standard solution of each compound. In all cases, negative mode provided a better signal-to-noise ratio.
Table 2: m/z, MS/MS transitions, optimal cone voltages and collision energies for investigated catechin derivatives.

<table>
<thead>
<tr>
<th>No</th>
<th>Compound</th>
<th>m/z</th>
<th>MS/MS transitions</th>
<th>Cone voltage (V)</th>
<th>Collision energy (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C</td>
<td>290.3 289.3 &gt; 109.3</td>
<td>40</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>EC</td>
<td>290.3 289.3 &gt; 109.3</td>
<td>40</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>AC</td>
<td>170.1 169.1 &gt; 125.2</td>
<td>30</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>CG</td>
<td>442.4 441.4 &gt; 169.3</td>
<td>40</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>ECG</td>
<td>442.4 441.4 &gt; 169.3</td>
<td>40</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>EGC</td>
<td>306.3 305.3 &gt; 125.3</td>
<td>40</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>GCG</td>
<td>458.4 457.4 &gt; 169.3</td>
<td>40</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>EGCG</td>
<td>458.4 457.4 &gt; 169.3</td>
<td>40</td>
<td>25</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 6 A shows the analysis of the eight catechins with MS detection operating in the tandem mode. This separation demonstrates the high quality chromatograms that can be obtained by UHPLC-MS/MS: all the peaks are base-line separated thanks to the combination of the LC system and MS detector. Commercial tea samples, commercially available on the market, were analysed with UHPLC-MS/MS instrument. Fig. 6 B shows that the separation is very good, even with real-world samples. Thanks both to the chromatographic separation and high sensitive detector it is possible to discriminate an additional peak appeared on the chromatogram before EGC. It has exactly the same parent ion and fragments as EGC, but different retention time. This method has to be validated for demonstrating its robustness and repeatability before using it for routine analyses.

Quantitation of each compound was obtained by calibration curves built (tab. 3) on five different concentrations in ranges depending on their abundance in the real-world samples. Quantitation had some problems since the real amount was always underestimated and the repeatability was not within the fixed limits.
General guidelines for method development of plant natural extracts to increase both efficiency and decrease time analysis

<table>
<thead>
<tr>
<th>Compound</th>
<th>Equation of calibration curve</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallic acid</td>
<td>$y=408.65x^{1.39}$</td>
<td>0.99</td>
</tr>
<tr>
<td>Catechins</td>
<td>$y=85.29x-5.19$</td>
<td>0.99</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>$y=79.01x-5.23$</td>
<td>1.00</td>
</tr>
<tr>
<td>Epigallocatechin</td>
<td>$y=127.67x-3.90$</td>
<td>0.99</td>
</tr>
<tr>
<td>Epigallocatechin gallate</td>
<td>$y=136.19x-39.71$</td>
<td>0.99</td>
</tr>
<tr>
<td>Catechin gallate</td>
<td>$y=167.71x-56.02$</td>
<td>0.99</td>
</tr>
<tr>
<td>Epicatechin gallate</td>
<td>$y=127.67x-3.90$</td>
<td>0.99</td>
</tr>
<tr>
<td>Gallocatechin gallate</td>
<td>$y=8.61x^2+88.59x-14.42$</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>$y=59.63x^{1.39}$</td>
<td>0.99</td>
</tr>
</tbody>
</table>

An internal standard, tyrosol, was therefore introduced to correct possible instrument fluctuations, but the final result was a further increase of RSD% (data not shown). Different known concentrations of standard compounds, chosen in relation with the estimated amount in the teas, were therefore added to all real samples to evaluate the eventual matrix effects. The sum of the markers naturally present in the drinks with the added ones did not correspond to the sum of the injected standard solutions plus teas injected separately. The quantity was lower in spiked teas, demonstrated a signal suppression probably due to a matrix effect. In fact, since the sample is composed by a large number of different compounds, the source is not able to ionize correctly all the molecules that arrive, so those not ionized are eliminated and therefore not quantified.

To overcome this problem, it would be necessary to work in the same condition, so the calibration curves should be built directly inside the real matrix, in this case tea, to avoid underestimation.

4 CONCLUSIONS
The aim of this study was to establish simple and general guidelines for a preliminary approach to the analysis of an unknown plant extracts and, eventually, to set and validate an analytical method with mass detector. Different parameters must be evaluated to find the best conditions of work. First, it is necessary to choose the more adapted organic solvent and vary its percentage so that all sample components have a $k$ included between 2 and 10. MeOH and ACN are the most used solvents, even if ACN is preferred because of its lower viscosity. If analytes present ionisable centres, mobile phase pH should be modified in order to work with non-dissociated molecules to obtain more symmetrical and more retained peaks. pH is an important tool to change selectivity by adding directly a low percentage of acid to both mobile phases or buffer to the aqueous mobile phase. Temperature is now considered an important parameter to set, not only to avoid peak shifts during the analyses, but also to modify selectivity of the chromatographic systems on the neutral molecules, to reduce backpressure affording higher flow rates or increase column length, to shorten analysis time and decrease the use of organic solvents, since water assumes behaviours similar to organic solvents. Reduction of retention time of 10% ca. is obtained with an increase of temperature of 15°C, while a decrease in backpressure of about 20%, is expected. The final step to tune selectivity or to improve separation is the change of column chemistry. The most used column is probably the C18, but this study confirmed that the presence of a carbammate in the side chain is a useful tool to correctly separate very close molecular structures while molecules with conjugated bonds or aromatic hydrocarbon cycles are successfully separated with phenyl columns.
UV detectors are the most popular, but, although they are very sensitive, linear, versatile and reliable, they are poorly diagnostic. MS detectors are more and more used for quality purposes. In this study, a quantitation approach was tested. Thanks to the high selectivity of tandem mass spectroscopy, it is possible to eliminate all the interfering peaks owing to the matrix and quantify correctly only the selected compounds. It is quite difficult to validate a quantitation method in complex samples, like plant extracts or food, since it is difficult to find a certified reference sample on which to build calibration curves, suitable internal standards, and signal suppression due to matrix effect is quite common.

5 REFERENCES

3.1.2 High throughput qualitative analysis of polyphenols in tea samples by ultra-high pressure liquid chromatography coupled to UV and mass spectrometry detectors

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ABSTRACT

The analysis of polyphenols in tea extracts is important due to their potential health benefits. Therefore, efficient and high throughput analytical methods have been developed for the separation of seven predominant polyphenols, also known as catechin derivatives, present in tea extracts. Columns packed with sub-2 μm particles operating at elevated pressure (UHPLC strategy) were selected to improve chromatographic performance. The potential of UHPLC–UV was demonstrated with baseline resolution of all standard catechins in only 30 s using a 50 mm column packed with 1.7 μm particles. When dealing with real samples such as tea extracts, however, longer columns of up to 150 mm in length were employed to enhance the separation of catechin derivatives and other constituents within the tea samples while maintaining an acceptable analysis time. Two strategies based on 2-D experiments were proposed to clearly identify catechins. Firstly, a liquid–liquid extraction procedure was added prior to the UHPLC–UV analysis to decrease the complexity of the sample. Secondly, UHPLC was coupled to ESI-MS/MS to attain sufficient sensitivity and selectivity between catechin derivatives and other constituents of tea extract. These two strategies were found extremely promising as a clear discrimination of catechins from the matrix could be attained.

KEYWORDS

Polyphenols; Catechins; Tea extract; LLE; UPLC; UHPLC; UPLC–MS/MS.

1. INTRODUCTION

Green and black teas are the most widely consumed beverages worldwide. Their potential health benefits, such as inhibitory effects against cancer and prevention of cardiovascular disease, have been extensively documented on a scientific basis [1]. It is generally believed that catechin flavonoids, whose content is around 60 mg/g of the dry leaf weight [2], possess antioxidant properties and biological activity responsible for the claimed therapeutic activity of tea [3] and [4]. For this reason, it is important to develop efficient analytical methods able to assess the nature and amount of catechins in various tea extract samples. Reverse-phase LC carried out on C8- or C18-bonded silica columns and coupled with UV, UV–DAD or electrochemical detection remains the most widespread method for the determination of polyphenols contained in plant and biological samples [5], [6], [7] and [8]. Recently published studies have reported analytical methods that require 20–60 min of analysis time per sample [5], [7], [8], [9] and [10] or between 20 and 40 min when only 5–10 compounds of interest have to be
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simultaneously measured [7], [8], [11], [12] and [13]. Furthermore, when more detailed structural information is required to identify and confirm molecular structures of unknown compounds, or when quantitative analysis has to be performed, RPLC methods can be coupled to electrospray mass spectrometry (ESI-MS) to take advantage of its high sensitivity and selectivity [7], [8], [13] and [14].

While providing satisfactory resolution, the above-reported RPLC methods for catechin analysis appear to have not taken full advantage of recent advances in liquid chromatography [15], [16] and [17]. Indeed, it could be beneficial to further improve chromatographic performance in terms of throughput and/or resolution particularly when numerous complex tea extracts have to be analyzed. In this context, a recent study shows the possibility of carrying out a separation of six epicatechin derivatives in 5–10 min using a conventional 100 mm, 3.5 μm RPLC material at an elevated flow rate (1.2 mL/min) [18]. Even if the authors claimed that the proposed method is repeatable, sensitive and can be used on a conventional HPLC instrument, the selectivity between the investigated compounds and other analytes contained within a real matrix (tea extract) is obviously too limited, and this makes an unambiguous determination and quantification of epicatechins difficult.

As an alternative, it would be interesting to evaluate the use of columns packed with sub-2 μm particles in conjunction with dedicated instrumentation able to withstand pressures of 1000 bar (ultra-high pressure liquid chromatography, UHPLC) [19] and [20]. This technology has become available from several providers [20] and [21] and has shown some clear benefits in terms of analysis time, resolving power, solvent consumption and, to a lesser extent, sensitivity [23], [24] and [25]. On the other hand, the main drawback for the use of columns packed with small particles is the generated backpressure, which is inversely proportional to the particle size, \( \text{dp}^3 \) when operating at the optimum mobile phase velocity according to Darcy's law [26]. This makes mandatory the use of dedicated instrumentation. Up to now, only a few applications using UHPLC have been reported for the analysis of catechins and with MS as detector [27], [28] and [29]. In the first study [27], the authors evaluated the amount of only two catechins, namely catechin and epicatechin, in various commercial chocolate samples in 3 min. In the second study, Solich et al. demonstrated the possibility of successfully separating various standard phenolic compounds including catechins. The unambiguous discrimination of catechins in real samples, however, remains critical [28]. Finally, the most recent study [29] developed a method for the qualitative and quantitative determination of catechins with UHPLC–PDA.

The present paper reports the development of efficient and high throughput UHPLC–UV methods for the separation of the seven predominant catechins present in tea extracts and gallic acid. UHPLC was also coupled with ESI-MS operating in the tandem mode to attain sufficient sensitivity and selectivity together with unequivocal identification between catechin derivatives and other constituents of tea extracts.

2 EXPERIMENTAL

2.1. Chemicals and reagents

The standard catechin flavonoids (+)-catechin (99%, C), (−)-epicatechin (96%, EC), (−)-catechin gallate (100%, CG), (−)-epicatechin gallate (99.1%, ECG), (−)-gallocatechin gallate (99.9%, GCG), (−)-epigallocatechin gallate (95.1%, EGCG), (−)-epigallocatechin (98.2%, EGC) and also gallic acid (99%, AC) were all obtained from Sigma–Aldrich (Milan, Italy) and stored at −20 °C in darkness. A stock solution of these eight compounds at a concentration of 0.5 mg/mL was initially prepared in pure methanol, and working standard solutions were obtained by appropriate dilution with pure water. Chemical structures of the eight investigated catechins are reported in Fig. 1. Additional chemicals such as ether (99%), ethyl acetate (99.5%) and butanol (98%) used
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for the liquid–liquid extraction procedures were purchased from Sigma–Fluka (Buchs, Switzerland).

Fig. 1. Chemical structures of the eight investigated polyphenols.

For the UHPLC–UV experiments, acetonitrile was of HPLC gradient grade from Panreac Quimica (Barcelona, Spain), and formic acid was obtained from SDS (Peypin, France). For the UHPLC–MS/MS experiments, formic acid and acetonitrile (ACN) were of ULC/MS grade and purchased from Biosolve (Valkenswaard, Netherlands). Finally, water was obtained from a Milli-Q Water Purification System from Millipore (Bedford, MA, USA).

2.2. Preparation of tea extract
Lipton brand black “tea time finest Ceylan” tea packaged in tea bags (2 g of tea per bag) was purchased at a local grocery store.

2.2.1 Preparation of a conventional tea infusion
A brew of black tea was prepared following the instructions provided on the package by pouring 200 mL of boiling water into a glass flask and dipping a 2 g tea bag for 5 min. After cooling to ambient temperature, the solution was filtered through a nylon filter (0.45 μm × 47 mm from Millipore). The tea extract was kept frozen at 4 °C until the analysis. Then, this sample was directly injected into the UHPLC–UV system and subjected to a 10-fold dilution with pure water before the UHPLC–MS analysis.

2.2.1. Liquid–liquid extraction procedure
Before the purification, all tea samples were filtered through a nylon filter (0.45 μm × 47 mm). Then, 10 mL of the filtered tea sample was extracted 3-fold with 10 mL of organic solvent (ether, butanol or ethyl acetate). After vigorous stirring, the two phases were allowed to settle for couple of minutes. The aqueous layer (lower part) was recovered in a glass flask and further analyzed. The organic layer (upper part) was recovered and evaporated to dryness using a nitrogen stream to avoid the peak broadening that is always observed when a pure organic
solvent is used for dilution purposes. The dry residue was subsequently dissolved in pure water before injection into the UHPLC system.

2.3. INSTRUMENTATION

2.3.1. UHPLC–UV experiments
UHPLC–UV experiments were performed on a Waters Acquity UPLC system (Milford, MA, USA). This instrument included a binary pumping system with a maximum flow rate of 2 mL/min, an auto-sampler with an injection loop volume of 2 or 5 μL used under full loop conditions, a UV–vis programmable detector and a column manager that included a column oven set at 30 °C. Data acquisition, data handling and instrument control were performed using the Empower Software v2.0. The UV detector time constant and data sampling rate were adjusted and could vary between 25–100 ms and 20–80 Hz, respectively, to obtain signals of highest quality even with ultra-fast separation. The detector wavelength was set at 265 nm as the best compromise to reach maximum absorbance for the compounds of interest.

2.3.2. UHPLC–MS/MS experiments
UHPLC–MS/MS experiments were performed on the same Waters ACQUITY UPLC system but hyphenated with a triple quadrupole (TQD) mass spectrometer from Waters. The TQD operated at a single mass resolution of m/z 0.7 FWHM and possessed an upper mass limit of m/z 2000. The ESCi® ionization source was used in the ESI negative mode, and ionization parameters, cone voltages and collision energies were optimized by infusing each compound (1 μg/mL) in 50:50 ACN:water plus 0.1% formic acid at a flow rate of 600 μL/min. Optimal cone voltage and collision energies values are summarized in Table 1. The capillary voltage and the source extractor voltage were set at 3000 and 3 V, respectively. The source temperature was maintained at 140°C, the desolvatation gas temperature and flow at 400°C and 800 L/h, respectively, and the cone gas flow at 50 L/h. MS/MS detection was carried out in the SRM mode, and the transitions are also indicated in Table 1. The collision gas flow was set at 0.2 mL/min of argon, and the entrance and exit potentials were adjusted to 1 and 0.5 V, respectively. Data acquisition, data handling and instrument control were performed using the Masslynx v4.1 software.

Table 1. m/z, MS/MS transitions, optimal cone voltages and collision energies for investigated catechin derivatives.

<table>
<thead>
<tr>
<th>No</th>
<th>Compound</th>
<th>m/z</th>
<th>MS/MS transitions</th>
<th>Cone voltage (V)</th>
<th>Collision energy (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C</td>
<td>290.3</td>
<td>289.3 &gt; 109.3</td>
<td>40</td>
<td>25</td>
</tr>
<tr>
<td>2</td>
<td>EC</td>
<td>290.3</td>
<td>289.3 &gt; 109.3</td>
<td>40</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>AC</td>
<td>170.1</td>
<td>169.1 &gt; 125.2</td>
<td>30</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>CG</td>
<td>442.4</td>
<td>441.4 &gt; 169.3</td>
<td>40</td>
<td>25</td>
</tr>
<tr>
<td>5</td>
<td>ECG</td>
<td>442.4</td>
<td>441.4 &gt; 169.3</td>
<td>40</td>
<td>25</td>
</tr>
<tr>
<td>6</td>
<td>EGC</td>
<td>306.3</td>
<td>305.3 &gt; 125.3</td>
<td>40</td>
<td>25</td>
</tr>
<tr>
<td>7</td>
<td>GCG</td>
<td>458.4</td>
<td>457.4 &gt; 169.3</td>
<td>40</td>
<td>25</td>
</tr>
<tr>
<td>8</td>
<td>EGCG</td>
<td>458.4</td>
<td>457.4 &gt; 169.3</td>
<td>40</td>
<td>25</td>
</tr>
</tbody>
</table>

2.3.3. Stationary phases
Separations were carried out on various analytical columns: a Hypersil Gold C18 (50 mm × 2.1 mm I.D., 1.9 μm) column provided by Thermo Fisher Scientific (Runcorn, UK), an Acquity BEH C18 (50 mm × 2.1 mm I.D., 1.7 μm) column, an Acquity BEH Shield RP18 (50, 100 and 150 mm × 2.1 mm I.D., 1.7 μm) column and an Acquity BEH phenyl (50 mm × 2.1 mm I.D., 1.7 μm) column. All of these columns were provided by Waters. The solvent system A = 0.1 vol.% formic acid – water; B = 0.1 vol.% formic acid – acetonitrile was used in the entire study.
2.4. HPLC modeling software

The optimal conditions for each sub-2 μm stationary phase were found thanks to HPLC modeling software (Osiris 4.1.1.2, Datalsys, Grenoble, France). For this purpose, the eight analytes were individually injected using two gradient runs that differ in slope, namely 2–40% B linear gradient in 14 and 4.8 min, respectively. Optimal conditions for separation with all stationary phases were determined for $k_{\text{min}}$ equal to 0.2 (no peak eluted before 0.3 min) and $k_{\text{max}}$ equal to 30 (no peak after 3.8 min).

3  RESULTS AND DISCUSSION

3.1. Determination of catechins by UHPLC–UV

There is a wide variety of polyphenols present in green or black tea extract. This study was dedicated to the determination of catechin flavonoids in particular, however, because it has been demonstrated that these compounds possess rather potent antioxidant properties and a significant degree of bioavailability, which results in beneficial health effects [1], [3] and [4].

3.1.1. Comparison of various columns packed with sub-2 μm particles

Despite some successful attempts in the analysis of catechins using capillary zone electrophoresis [30], micellar electrokinetic chromatography [31], gas chromatography after derivatization [32] or thin-layer chromatography [33], the method of choice, which encompasses approximately 80% of the literature, remains RPLC with UV absorbance detection. An RPLC–UV baseline separation of the eight most abundant catechins was first published by Goto et al. [34] in which a C18 stationary phase and a complex gradient system made of water, acetonitrile and phosphoric acid were used. This interesting study demonstrated that four catechins (EGC, EGCG, EC and ECG) were predominant in tea extract while the other catechins were only present in minor amounts. In 1998, Dalluge et al. [35] showed that acidic conditions were mandatory to both attain a complete resolution of the catechins and eliminate peak tailing. The same study [35] also demonstrated that the complete separation of catechins was column-dependent and concluded that endcapped, deactivated, monomeric C18 columns were preferable. Therefore, four different endcapped, deactivated columns packed with sub-2-μm particles from two different providers were tested in acidic conditions to attain the baseline separation of seven important catechins and gallic acid. Among these supports, a conventional C18 material (Hypersil Gold C18), a hybrid BEH C18 support (Acquity BEH C18), a hybrid BEH RP18 support with a polar (carbamate) embedded group (Acquity BEH Shield RP18) and a hybrid BEH phenyl material (Acquity BEH phenyl) were initially selected. A systematic procedure was employed to determine the best conditions for the separation of the eight compounds of interest. This operation essentially consists of performing two gradient runs that differ in slope with the eight compounds and the four supports using a water–acetonitrile mobile phase with 0.1% formic acid. Then, the data were computed in optimization software to model the behavior of each compound in the whole composition range. Fig. 2 presents the optimal chromatograms obtained with the four stationary phases. Firstly, it is worth mentioning that the elution order of non-epi and epi forms was identical for all investigated columns and was in close agreement with previous studies [36] and [37]. Indeed, epi forms with gallate (EGCG and ECG) were eluted prior to non-epi forms with gallate (GCG and CG), and a non-epi form without gallate (C) was eluted prior to non-epi forms without gallate (EGC and EC).
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Fig. 2. Optimal UHPLC chromatograms of an eight standard polyphenols mixture at 20 μg/mL obtained with various RP columns packed with sub-2μm particles at 30 °C. Numbers correspond to Fig. 1. F = 500 μL/min and a gradient procedure was used with A as pure water and B as ACN. 0.1% formic acid was present in both solvents. (A) Acquity BEH C18 50 mm × 2.1 mm ID, 1.7 μm column, gradient 11–16.6%B in 3.52 min. (B) Hypersil GOLD C18 50 mm × 2.1 mm ID, 1.9 μm column, gradient 10–16.3%B in 3.52 min. (C) Acquity BEH phenyl 50 mm × 2.1 mm ID, 1.7 μm column, gradient 16.8–23.4%B in 0.6 min. (D) Acquity BEH Shield RP18 50 mm × 2.1 mm ID, 1.7 μm column, gradient 13.3–22.2%B in 3.46 min. 1: C, 2: EC, 3: AC, 4: CG, 5: ECG, 6: EGC, 7: GCG, 8: EGCG.

3.1.2. Ultra-fast and highly efficient separations of catechins

One of the main advantages of UHPLC technology is related to the possibility of attaining either ultra-fast or high resolving power separations. Indeed, with the Van Deemter curves obtained for
columns packed with small particles [26] and the elevated backpressure attainable (up to 1000 bar in UHPLC instrumentation [22]), it is possible to tune column geometry, the mobile phase flow rate and other chromatographic conditions to attain fast or highly efficient separations.

Because the separation presented in Fig. 2D presents an elevated selectivity, it is possible to further increase the mobile phase flow rate, the value of % B initial and the gradient slope while maintaining acceptable separation. In this perspective, an 18–30% ACN gradient in 0.3 min was carried out at a flow rate of 1 mL/min to speed up the original separation as much as possible. The final chromatogram presented in Fig. 3A shows a baseline separation of the eight phenolic compounds in only 30 s with a minimal resolution of 1.72 for C and EC (peaks number 1 and 2). For this separation, the generated backpressure was around 850 bar, and this remained acceptable even for routine use of the method.

On the other hand, the determination of some minor compounds contained within a complex matrix such as tea extract, which possesses hundreds of constituents, requires a high resolving power. Thus, the conditions used for Fig. 3A cannot be applied to real tea samples. Additionally, even if the separation in Fig. 2D presents a high selectivity for catechins, the global resolving power is still too limited to deal with real samples. The performance in gradient mode can be estimated with the peak capacity (P), which is the number of peaks that can be separated in a given time window. The latter is based on the gradient time (t_{grad}) and peak width at the baseline in time units (W_t) according to the following equation:

\[ P = \frac{t_{grad}}{W_t} \]
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\[ P = \frac{t_{\text{grad}}}{W_t} + 1 \]  

(1)

For the separation reported in Fig. 2D, the peak capacity was equal to 35. To increase peak capacity, longer columns packed with small particles have been tested with extended gradient time. However, to avoid changes in selectivity, the basic rules for method transfer in gradient mode, which have been presented elsewhere [38], have been strictly applied. In Fig. 3B, a 100 mm × 2.1 mm, 1.7 μm column was used at a flow rate of 0.5 mL/min. As the column length varied, the gradient time was scaled accordingly (3.46 vs. 7.05 min) while the initial and final composition remained identical. In Fig. 3B, the quality of the separation was significantly improved and peak capacity was 2-fold higher (70). Finally, the column length was further increased to 150 mm and the gradient time extended to the maximal acceptable value (30 min). The obtained chromatogram is presented in Fig. 3C. Under these conditions, the value of \( P \) was further increased to 85 in 20 min. Therefore, the gain in peak capacity between Fig. 3C and B, which was equal to 20%, was quite limited in proportion to the increase in analysis time (4-fold) and to the generated backpressure. This observation is in good agreement with recent UHPLC studies [39], [40] and [41] that show the longest column does not necessarily provide an important increase in gradient performance as the latter depends on both isocratic efficiency and the column dead time, which is elevated with longer columns operating at low flow rate.

Eqs. (2) and (3) show the interdependency of peak capacity with isocratic efficiency (\( N \)) and column dead time (\( t_0 \)) [42] and [43]:

\[ P = 1 + \frac{\sqrt{N}}{4} \cdot \frac{1}{b+1} \ln \left( \frac{b+1}{b} e^{S \cdot \Delta \Phi} - \frac{1}{b} \right) \]  

(2)

\[ b = \frac{t_0 \Phi S}{t_{\text{grad}}} \]  

(3)

where \( t_{\text{grad}} \) is the gradient time, \( \Delta \Phi \) is the change in solvent composition during the gradient, ranging from 0 to 1, and \( S \) is a parameter (slope of the logarithmic plot: \( d(log k)/d\Phi \)) related to the solute nature, molecular weight and organic modifier nature.

In conclusion, the separation presented in Fig. 3B represents the best compromise between throughput and resolving power for the separation of the seven-catechin derivatives and gallic acid.

3.1.3. Application to commercial tea extract

When dealing with standard polyphenols, the UHPLC–UV method provides excellent selectivity and resolution as demonstrated above. This proposed strategy was thus applied to the determination of catechin derivatives in real tea samples.

To illustrate the complexity of the matrix, a real tea sample infused for 5 min was injected into the UHPLC–UV system using the methods described in Fig. 2 and Fig. 3, which correspond to analysis times of 2, 7 and 24 min, respectively. The corresponding profiles are presented in Fig. 4 (bold chromatograms) with a simultaneous overlay of the separations of the eight standard catechins (light chromatograms). It appears from these figures that tea extracts are very complex matrices, and, as expected, the different proposed methods do not present enough resolving power to easily discriminate catechins contained in tea samples.
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Despite these critical separations, it is possible to draw some qualitative conclusions regarding the content of catechins in our commercial tea sample. Firstly, it is possible to assess that there was an important amount of EGCG (peak 8 at 1.23 min in Fig. 4A) and ECG (peak 5 at 1.66 min in Fig. 4A) in the tea extract as the corresponding peaks in the three chromatograms were quite significant. It is a bit more difficult to make the same conclusion on the presence of EC (peak 2 at 0.92 min in Fig. 4A) and EGC (peak 6 at 0.62 min in Fig. 4A) because quantities were lower. The small peaks were observed identically on the three chromatograms and confirmed by standard injection. These results are in good agreement with a previous study [2], which demonstrated that EGC, EGCG, EC and ECG were the predominant catechins in tea samples. Regarding AC (peak 3 at 0.39 min in Fig. 4A), its presence in large quantity was confirmed by the three chromatograms of Fig. 4, and this is logical as it is a degradation product of catechin derivatives. Finally, regarding C (peak at 0.78 min in Fig. 4A), GCG (peak at 1.42 min in Fig. 4A) and CG (peak at 1.89 min in Fig. 4A), there is no consensus between results provided by the three profiles presented in Fig. 4. Indeed, there is no evidence for these three compounds in Fig. 4A and C while there were some important peaks for these three catechins in the chromatogram of Fig. 4B. This clearly demonstrates the limitations of the UHPLC–UV strategy for the qualitative evaluation of catechins and the difficulty of carrying out the quantitative evaluation of complex tea extracts. To avoid these problems, two strategies were investigated: (i) addition of a selective sample preparation, namely LLE prior to the UHPLC–UV separation (discussed in Section 3.2) and (ii) switching from the universal UV to the selective MS detector (discussed in Section 3.3).

3.2. Determination of catechins by LLE–UHPLC–UV
A sample preparation step was added to the analytical procedure to decrease the complexity of the tea extract samples and to obtain a better evidence about the presence or absence of catechin derivatives. Various generic, simple liquid–liquid extraction (LLE) procedures were evaluated
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prior to the UHPLC–UV analysis. After maceration of tea in pure water, LLE with butanol, ether, ethyl acetate..., were performed according to the literature [5], [44] and [45]. These procedures were compared in term of recoveries, calculated as the ratio of catechin content in organic solvent and total catechin content in both water and organic solvent, for the seven investigated catechins. The recovery values were obtained with the selective and sensitive UHPLC–MS/MS method described in Section 3.3. Fig. 5 shows the results obtained for all catechins using the three different extracting solvents. Firstly, ether is too nonpolar for extracting catechins, and it was rejected because all catechins were recovered in the aqueous phase. The results for ethyl acetate or butanol were very close with recoveries between 93.7% and 100% for ethyl acetate and 92% and 100% for butanol. Only the recoveries of CG and GCG were below these values with both solvents. The lower values observed for these compounds were attributed to their rather low concentration in real tea samples, which are below the limit of quantitation (LOQ). This can make the determination of these compounds in both phases inaccurate and imprecise.

Fig. 5. Recoveries for the seven-catechin derivatives using three different extraction procedures. Recoveries were determined with the UHPLC–MS/MS method presented in Fig. 8A.

Considering these observations, ethyl acetate was selected as the extraction solvent, because it was easier to use than butanol, which can lead to mixing of the two phases. The two chromatograms presented in Fig. 6 correspond to the direct injection of a commercial tea extract infused for 5 min (light chromatogram) and the organic fraction of the same extract after a liquid–liquid extraction with ethyl acetate (bold chromatogram). This separation clearly demonstrates the importance of adding a sample preparation step such as LLE with ethyl acetate prior to the analysis. From this separation, it is now possible to confidently identify catechins present in tea samples. In the tested tea extract, the abundance of catechin derivatives can be classified as follows: EGC > EC > EGCG > ECG > GCG > C > CG.
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3.3. Determination of catechins by UHPLC–MS
Another strategy was alternatively proposed to further enhance the UHPLC–UV separation of catechins in real tea extracts. Even if UV remains the gold standard detection mode for routine catechin determination, a more selective detector could be useful in some instance [46]. The addition of LLE prior to UHPLC–UV was found to be promising (Section 3.2) but, mass spectrometry (MS) was also successfully reported for catechin analysis to minimize problems associated with inadequate separation quality [2], [47] and [48].

3.3.1. Selectivity of UHPLC–MS and UHPLC–MS/MS towards catechins
In this study, the possibility of coupling UHPLC with MS detection, without any sample preparation, was evaluated for the determination of polyphenols. For the UHPLC–MS experiments, the seven-minute method (Fig. 3B) was selected as it presents the best compromise between analysis time and resolving power. The corresponding chromatograms are presented in Fig. 7. In these separations, real tea samples were diluted 10-fold with water before analysis to avoid contamination of the ESI source and eventual clogging of the heated capillary. The ESI source parameters (i.e., cone voltage, capillary voltage, source extractor voltage, source temperature, desolvatation gas temperature and flow and cone gas flow) were tuned by infusion experiments in the single ion monitoring (SIM) mode to attain the highest possible intensity of ions. In all cases, the negative mode provides a better sensitivity than the positive mode, and the [M–H]- adduct always presented the highest signal-to-noise ratio. Using the optimal ESI settings reported in Section 2.3, product ion scans were acquired with collision energies ranging between 10 and 60 eV, and the most intense transition was selected for each monitored precursor m/z. The optimal transitions and the corresponding collision energies are reported in Table 1.
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Fig. 7. UHPLC–MS/MS analysis of seven-catechin derivatives and gallic acid. Chromatographic conditions were similar to that of Fig. 3B. (A) Mixture of eight polyphenols standards at 1 μg/mL in the SRM mode. (B) 10-fold diluted commercial tea extract infused for 5 min with polyphenols detected in the SIR mode (UHPLC–MS). (C) 10-fold diluted commercial tea extract infused for 5 min with polyphenols detected in the SRM mode (UHPLC–MS/MS). 1: C, 2: EC, 3: AC, 4: CG, 5: ECG, 6: EGC, 7: GCG, 8: EGCG.

The selected transitions were identical for both non-epi and epi form of an isomeric pair, and the major fragments were 109, 125 and 169 m/z. The catechin structures reported in Fig. 1 enable a discussion of the fragmentation pathways. First, the product ion 109 m/z was mainly observed for C and EC and should arise from the cleavage of a C–C bond to yield the 2-hydroxyphenol ion (109 m/z). The ion 125 m/z (109 m/z + OH) was the major ion for AC and EGC, but it was also observed on the product ion scans of CG, ECG, GCG and EGCG at high collision energy. This ion should correspond to the 2,3-dihydroxyphenol group common to all these molecules. Finally, the ion 169 m/z, which corresponds to a loss of gallate, was obviously the most intense fragment for the four catechins possessing a gallate moiety (CG, ECG, GCG and EGCG).

Fig. 7A presents the analysis of the eight standard catechins using MS detection operating in the tandem mode. This separation demonstrates the high quality chromatograms that can be attained in UHPLC–MS/MS. It is indeed impossible to assess the different catechins on the basis of MS response alone as the different epimers possess a similar m/z ratio and fragmentation pathways. This leads to identical MS/MS transitions. As the chromatographic separation of epimers was complete, however, the differentiation of catechin derivatives can be safely assessed in UHPLC–MS/MS. In this separation, the sensitivity was far better than that of UV with signal-to-noise (calculated with an internal algorithm from the Masslynx software) comprised between 200 for GCC and 1000 for AC at a concentration of 1 μg/mL.

A real sample of diluted tea extract was injected into the system to highlight the elevated selectivity of the MS device. Initially, the detector was used as a single quadrupole instrument operating in the SIM mode, and the chromatogram is reported in Fig. 7B. Under these conditions, numerous contaminants were observed on all channels, particularly for the corresponding m/z ratio of EGCG and ECG, and this led to limited sensitivity and selectivity. The low sensitivity and selectivity can obviously be attributed to the low resolution of the single quadrupole instrument, which is around 0.7–1 FWHM. The same tea extract sample was also analyzed with an MS instrument operating in the tandem mode. As observed in Fig. 7C, the selectivity was significantly improved, and the qualitative discrimination of each catechin was very straightforward based on their retention time and MS/MS transitions. Under these conditions, the sensitivity was excellent with signal-to-noise higher than 500 for AC in the 10-
fold diluted tea sample. Only a contaminant peak at 0.99 min was observed for the transition of EGC, but this peak possessed a different retention time from that of peak number 6. Similarly to the LLE–UHPLC–UV, it is also possible to establish a qualitative ranking between the different catechins present in our commercial tea sample using the UHPLC–MS/MS method. The abundance of catechins can now confidently be classified as follow: EGC > EC > EGCG > ECG > C > CG > GCG, by comparing the chromatogram of tea extract with that of standard catechins in pure water.

4 CONCLUDING REMARKS

The aim of this study was to show the possible benefits of using columns packed with sub-2 μm particles in ultra-high pressure conditions (UHPLC) for the qualitative determination of various catechin derivatives in tea extracts. After a careful selection of the most appropriate column chemistry (Acquity BEH Shield RP18), it was demonstrated that the separation of eight standard polyphenols could be achieved in about 30 s while maintaining sufficient resolution. When dealing with complex matrices such as tea extracts, however, which could possess hundreds of constituents, the resolving power becomes more important than throughput. For this reason, longer columns have been tested, and a good compromise was obtained with a 100-mm column using a gradient from 13% to 22.2% ACN in 7.05 min. In real tea extracts, the unambiguous UV identification of catechins always remains tedious, and quantitation also remains critical. For this reason, two procedures involving 2-D experiments were implemented. On the one hand, the complexity of tea extract samples was reduced with the help of a simple, rapid purification procedure. A liquid–liquid extraction with ethyl acetate as the organic solvent was employed prior to the UHPLC–UV analysis, and this demonstrated some evident benefits for catechin determination. On the other hand, UHPLC was coupled to MS/MS detection to attain a sufficient sensitivity and selectivity between catechin derivatives and other constituents of the tea extract sample. This strategy was found to be extremely well suited as selectivity remains excellent in the SRM mode even with complex tea extracts.

As shown in this paper, the two proposed strategies, namely LLE–UHPLC–UV (gold standard for routine analysis) and UHPLC–MS/MS, are very useful for an unambiguous qualitative determination of catechins in tea. A study about quantitation of catechins in tea by these two approaches is under way.
5 REFERENCES

3.2 High Efficiency Analyses
High efficiency is the main goal when unknown plant samples are analyzed. In each of them, it is possible to have both compounds belonging to different chemical classes and compounds with similar chemical structure, or even isomers to be separated. It is important to discriminate very close compounds, since it is possible that one isoform is more active than the other or even the responses are different.

The optimization of the analytical method is not always sufficient for a baseline separation of all the compounds present in a sample, but nowadays several new approaches can be employed for this purpose:

- sub-2 µm columns,
- core-shell columns,
- sensitive detectors,
- high temperature.

Sub-2 µm and core-shell columns are able to increase efficiency thanks to the improvements of the three parameters of van Deemter equation: the A, the B and the C term, compared to conventional 5 µm columns. High temperature influences many parameters of the mobile phase, permitting especially faster exchanges of the molecules between mobile and stationary phase and therefore leading to narrower peaks and then to increase efficiency.

Sensitive detectors are very useful to overcome limitations in chromatographic separations. It is therefore possible to work with higher flow rates by partially compromising the separation. However it is possible to gain an increasing amount of information about the molecules present in the sample, such as the exact molecular weight, molecular formula, etc.

In spite of the use of these new techniques, it is not always possible to reduce analysis time. Especially for analyses without MS detectors, it is important to have the highest efficiency to obtain a baseline separation to make possible a collection of the unknown peaks and when possible to analyze them in NMR for structure evaluation.

In this section two examples of high resolution analyses for different plant extracts with different detectors are reported.

In the first study, three matrices are analyzed both with core-shell and sub-2 µm columns to compare their performance by Qq-TOF/MS detector and then the efficiency was increased by coupling together up to three columns and by increasing temperature.

In the second study, poplar absolute was analyzed by der-GS-MS and HPLC-UV and then the method was optimized on a UHPLC system to increase the separation.
3.2.1 High resolution profiling of complex plant extracts by modern liquid chromatography strategies *

*to be submitted to an international journal

ABSTRACT

When analyzing natural products, it is mandatory to employ highly efficient approaches to separate a maximum number of peaks in a single chromatographic run. In this paper, various analytical strategies involving liquid chromatography were compared using three complex model plant extracts, namely rosemary (*Rosmarinus officinalis* L.), juniper (*Juniperus communis* L.) and gingko (*Ginkgo Biloba* L.). The performance of three different columns packed with sub-3 µm core-shell and two columns packed with fully porous sub-2 µm particles were compared in terms of retention, selectivity and peak capacity, using QqTOF/MS detection. The most retentive phases for all the extracts were the Poroshell and Acquity phases. The Kinetex phase was always the most different one in terms of selectivity for the three plant extracts. These differences in retention and selectivity were attributed to possible differences of silica matrix, bonding density and nature of end-capping. Finally, the best kinetic performance with plant extracts was systematically attained with the Ascentis material, followed quite closely by the Acquity phase. However, the peak capacities attained with three other phases, Kinetex, Poroshell and Hypersil Gold, were significantly poorer.

If mass spectrometry is not available, a suitable strategy with UV detection could be considered to further increase the chromatographic resolution by coupling several columns together and increasing temperature up to the upper limit of the column. Under such extreme conditions, peak capacity of more than 500 was reported using a column of 450 mm packed with sub-2 µm porous particles at 90°C.

KEYWORDS: plant extracts, high resolution analysis, core-shell, sub-2 µm, high temperature UHPLC.

INTRODUCTION

Over the last decades, the need for analytical laboratories to improve data quality, increase sample throughput or obtain high resolution analyses, has generated a considerable interest in new liquid chromatography strategies. In the case of natural products, the samples are very complex and could contain several hundreds of metabolites. Thus, to obtain reliable information, high resolution liquid chromatography is mandatory. In the last ten years, a number of different approaches has been proposed to improve chromatographic performance, including: i) monolithic supports, ii) high temperature liquid chromatography (HTLC), iii) columns packed with small particles (sub-2 µm) operating under ultra-high pressure conditions (UHPLC) and iv) columns packed with sub-3 µm core-shell particles [1,2].

Monolithic columns represent an innovative analytical approach to reach high efficiency in reasonable analysis time. Their shape could be associated to a coherent, rigid, single rod obtained by co-polymerization of silica or different organic polymers [3]. They are constituted by macropores and mesopores [4] and this particular structure allows to work at high flow rates without compromising efficiency and generating a too high backpressure [5,6]. Different studies show that the backpressure was comparable to that generated by a column, of identical dimension, packed with 11 µm particles and efficiency is equivalent to that of a column packed with 3 - 3.5 µm particles [3]. There are, however, some limitations in using monolithic columns: the stationary phase chemistries and dimensions are restricted and the chemical (pH and temperature) as well as mechanical (backpressure) stability of such columns remains limited [3-6].
Until recently, temperature has always had a minor role in HPLC method development and only a small number of applications have been published [7]. In reality, temperature has important effects on mobile phase viscosity, polarity, surface tension and solute diffusivity. As example, at elevated temperature, mobile phase viscosity is decreased, and so does the pressure drop, allowing to work at higher flow rates (high throughput separation) or with longer columns (highly efficient separation) [8,9]. HTLC presents also some drawbacks: a limited number of stationary phases are stable at elevated temperature [9,10], the compound stability could be an issue and it is finally mandatory to have some specific devices to control mobile phase temperature [8,9,11].

Another option to achieve high resolution separations is to use columns packed with fully porous small particles. Particle diameter reduction results in improvement of efficiency, solute mass transfer and optimum velocity [12,13]. It is therefore possible to work at higher flow rates than the optimum one without loss in performance, as the C-term region of the Van Deemter curve is flatter [14]. However, forcing liquid through columns packed with small particle increases dramatically backpressure and this requires chromatographic systems compatible with pressures up to 1200-1300 bar [15]. Moreover, peaks are narrower than in conventional HPLC, so the whole instrument must be optimized, reducing the injection cycle time, dwell volume, system dead volume and increasing the acquisition rate of the detector [16,17].

Core-shell technology consists of using columns packed with particles formed by a solid core covered by a thick layer of porous silica shell [18]. Compared to fully porous particles of the same diameter, the diffusion path of the solutes is reduced, because of the solid core [14, 19] and the particle size distribution and packing density is improved leading to a smaller A term of the Van Deemter equation [20]. Columns packed with core-shell particles allow to reach speed and efficiency values quite similar to those of columns packed with sub-2 µm particles [21-23]. However, the feature that mostly helped to their success is the two-fold lower generated backpressure compared to sub-2 µm columns, that leads to obtain high resolution analysis even on “conventional” HPLC instruments [21,23].

Natural products have provided, since a long time, the inspiration of a large number of active molecules in medicine. This success is probably linked to their high chemical diversities, but they represent even the biggest challenge in terms of separation, detection and characterization. For these reasons, it could be of interest to consider the last developments in separative and detection techniques to improve the analysis of complex matrices or to find new interesting compounds [24-28]. Some recent publications show the potential of sub-2 µm columns and UHPLC systems coupled together with the high sensibility of mass spectrometers for different natural samples or phytopharmaceuticals, such as Ginkgo Biloba, Arabidopsis thaliana, Panax notogingenseng, etc. [25,28-30], while core-shell technology has not been so deeply investigated until now. In fact, only few articles described their chromatographic potential in natural product separations [31-36].

The aim of this study was to test different strategies to improve the separation of complex plant samples. For this purpose, three model extracts of different complexity were chosen, namely rosemary (Rosmarinus officinalis L.), juniper (Juniperus communis L.) and gingko (Ginkgo Biloba L.). Five different columns of 150 mm length, including three sub-3 µm core-shell and two fully porous sub-2 µm columns, were tested and their chromatographic performance compared. Analyses were performed in UHPLC coupled with UV and QqTOF/MS detectors to highlight the limits of each detector.
1. EXPERIMENTAL

2.1 Chemical and reagents
The solvent used for the extraction of the *Juniperus communis* L. was methanol (MeOH). Dissolution and dilution solvents were MeOH and ethanol (EtOH) respectively for juniper, ginkgo and rosemary. For UHPLC-QqTOF/MS and UHPLC-UV experiments acetonitrile (ACN) and water (H$_2$O) were of LC-MS grade from Biosolve (Valkenswaard, the Netherlands). Aqueous and organic mobile phases were acidified by 0.1 volume % of formic acid LC-MS grade from Biosolve (Valkenswaard, the Netherlands). Standardized *Rosmarinus officinalis* L. and *Ginkgo Biloba* L. extracts were gently offered by Robertet SA (Grasses, France) and Indena (Milan, Italy), respectively. A stock solution at 5 mg/mL of each sample was initially prepared.

2.2 Preparation of *Juniperus communis* L. needle extract
Dry juniper needles were first grinded. 10 mL of MeOH were added to 1 g of matrix to extract the fraction of interest by ultrasound for 15 minutes. This process was repeated for three times collecting every time the extraction solvent together and refilling with fresh one. The three fractions were filtered on paper filter and then evaporate to dryness by rotatory evaporation. The reconstituted sample in MeOH was filtered, before dilution and UHPLC injections, on PTFE filters 0.45µm x 4 mm.

2.3 Instrumentation

2.3.1 UHPLC-QqTOF/MS experiments
Experiments in UHPLC-QqTOF/MS were performed on the Waters Acquity UPLC system (Milford, MA, USA). This instrument included a binary pumping system with a maximum flow rate of 2 mL/min, an auto-sampler with an injection loop volume of 5 µL used in full loop conditions, a column manager that include a column oven set at 40°C and time-of-flight mass spectrometer. A Xevo-TOF-1 time-of-flight mass spectrometer from Waters with an electrospray ionization source was used. ESI conditions were as follow for negative ionisation mode: capillary voltage of 3000 V, cone voltage of 20 V, MCP detector voltage of 2650 V, source temperature of 150°C, desolvatation temperature 500°C, cone gas flow of 20 L/h and desolvatation gas flow of 1000 L/h. Detection was performed over a m/z range of 100-1000 in the centroid mode, with a duty cycle of 0.3 s. All analyses were acquired using the programmed dynamic range enhancement (pDRE) technology and an independent reference lock-mass ion infused through LockSpray™ interface to ensure accuracy and reproducibility. A solution of leucine-enkephalin (m/z 556.2771) at a concentration of 0.2 ng/mL was used as the reference compound and was infused at a flow rate of 5 µL/min. LockSpray frequency was set at 20 s and averaged over 5 spectra. Data acquisition, data handling and instrument control were performed by MassLynx Software v4.1.

2.3.2 UHPLC-UV experiments
The same UHPLC instrument was used for UHPLC-UV analyses. Injection loop volume of 20 µL was used in partial loop with needle overfill conditions and the instrument included a UV-VIS programmable detector and a column manager with a column oven set at 40 or 90°C. Data acquisition, data handling and instrument control were performed by Empower Software v2.0. To obtain signals of highest quality, the UV detector time constant and data sampling rate were set at 100 ms and 20 Hz, respectively. The wavelength was set at 270 and 370 nm, respectively for junipers and Ginkgo as the best compromise to reach maximum absorbance for all the compounds present in the sample.
2.4 Columns

Separations were carried out on various analytical columns: Hypersil Gold C18 (150 x 2.1 mm ID, 1.9 µm) provided by Thermo Fisher Scientific (Runcorn, UK); Acquity BEH C18 (150 x 2.1 mm ID, 1.7 µm) provided by Waters (Milford, MA, USA), Poroshell C18 (150 x 2.1 mm ID, 2.7 µm) provided by Agilent (Santa-Clara, CA, USA), Kinetex C18 (150 x 2.1 mm ID, 2.6 µm) provided by Phenomenex (Torrance, CA, USA) and Ascentis Express C18 (150 x 2.1 mm ID, 2.7 µm) provided by Supelco (Bellefonte, PA, USA). The following solvent system: A = 0.1 vol.% formic acid - water; B = 0.1 vol.% formic acid – ACN was used in the whole study.

2. RESULTS AND DISCUSSION

Because of the complexity of a plant extract sample and the number of isomers that can be encountered, there is a need for chromatographic method providing high resolution. To meet this requirement, two different strategies were evaluated. The first one consists in combining high resolution LC and QqTOF/MS detection. The second one deals with the use of ultra-high resolution (use of very long and efficient columns) liquid chromatography, combined with a simple UV detection for control quality and to overcome the eventual lack of MS detection.

3.1. High resolution LC-QqTOF/MS for plant extract analysis

For this part of the work, three model plant extracts were considered, namely rosemary (*Rosmarinus officinalis* L.), juniper (*Juniperus communis* L.) and gingko (*Ginkgo Biloba* L.). These three extracts were selected since they present an important number of metabolites with very different physico-chemical properties (polarity, chemical functions, pKa and size). To attain high resolution in LC, various solutions can be envisaged and among them, the use of long (150 mm) columns packed with core-shell particles could be a suitable strategy. For this reason, three columns of this type were selected, namely the Agilent Poroshell C18, Phenomenex Kinetex C18 and Supelco Ascentis Express C18, with particle sizes ranging between 2.6 and 2.7 µm and shell thickness comprised between 0.35 and 0.5 µm. These columns withstand pressure up to 600 bar, according to the provider, even if it has recently been demonstrated that the Kinetex one could be employed up to 1200 bar [37]. For comparison purpose, two UHPLC columns of the same length and packed with fully porous sub-2 µm particles were also considered, namely the Thermo Hypersil Gold C18 and Waters Acquity BEH C18. These columns are compatible with pressure up to 1000 bar and 1200 bar, for the Hypersil Gold and the Acquity, respectively. To have a fair comparison of retention properties, kinetic efficiency, selectivity and overall resolution of these five different stationary phases, all the analyses were performed using the same instrumentation, namely a UHPLC-QqTOF/MS device.

The five chromatograms corresponding to the rosemary have been reported in Figure 1. Rosemary is an evergreen shrub and has been used in folk medicine for centuries. Nowadays, it is possible to attribute its antioxidant effects to phenolic compounds, that, in rosemary, belong to different classes: diterpenes, flavonoids and phenolic acids.[38; 39]. Interest for its properties has recently increased to evaluate the possibility to substitute synthetic, such as BHA and BHT, by natural antioxidants [40]. Moreover biological activities are also reported, such as antimicrobial, anti-inflammatory, antiviral, diuretic, antiulcerogenic and cancer prevention functions [39,41].
Thanks to the QqTOF/MS device, it was possible to find the position of a few known markers for this plant extract (identified on the chromatograms as peaks 1 to 5), based on exact mass measurements. Between the different stationary phases, the chromatograms look quite similar in terms of retention, selectivity and peak widths. In terms of retention, the differences between columns were not drastic probably because the experiments were conducted under gradient elution conditions (highest difference between $t_r$ on the different phases was equal to ~7%). However, it appears that the Poroshell and Acquity were the most retentive phases. This observation is in agreement with a work recently published by our group which also demonstrates that these two phases were more retentive than other type of core-shell material [23]. The observed differences between stationary phases can be attributed to changes of carbon load, pore diameter, surface coverage nature of the endcapping or bonding process.
Table 2: Selectivity of the reference peaks of the three samples on the five columns based on figs. 1, 3 and 4.

<table>
<thead>
<tr>
<th></th>
<th>Poroshell</th>
<th>Ascentis</th>
<th>Kinetex</th>
<th>Hyperion gold</th>
<th>Acquity</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \alpha ) 1-2</td>
<td>1.72</td>
<td>1.74</td>
<td>1.71</td>
<td>1.70</td>
<td>1.71</td>
</tr>
<tr>
<td>( \alpha ) 2-3</td>
<td>1.44</td>
<td>1.45</td>
<td>1.42</td>
<td>1.43</td>
<td>1.45</td>
</tr>
<tr>
<td>( \alpha ) 3-4</td>
<td>1.14</td>
<td>1.15</td>
<td>1.13</td>
<td>1.14</td>
<td>1.15</td>
</tr>
<tr>
<td>( \alpha ) 4-5</td>
<td>1.07</td>
<td>1.07</td>
<td>1.07</td>
<td>1.07</td>
<td>1.07</td>
</tr>
<tr>
<td>( \alpha ) 1-2</td>
<td>1.89</td>
<td>1.94</td>
<td>1.99</td>
<td>1.87</td>
<td>1.87</td>
</tr>
<tr>
<td>( \alpha ) 2-3</td>
<td>1.08</td>
<td>1.08</td>
<td>1.02</td>
<td>1.09</td>
<td>1.12</td>
</tr>
<tr>
<td>( \alpha ) 3-4</td>
<td>1.55</td>
<td>1.34</td>
<td>1.49</td>
<td>1.67</td>
<td>1.58</td>
</tr>
<tr>
<td>( \alpha ) 4-5</td>
<td>1.15</td>
<td>1.36</td>
<td>1.29</td>
<td>1.06</td>
<td>1.09</td>
</tr>
<tr>
<td>( \alpha ) 5-6</td>
<td>1.66</td>
<td>1.66</td>
<td>1.56</td>
<td>1.64</td>
<td>1.67</td>
</tr>
<tr>
<td>( \alpha ) 1-2</td>
<td>2.38</td>
<td>2.38</td>
<td>2.42</td>
<td>2.20</td>
<td>2.40</td>
</tr>
<tr>
<td>( \alpha ) 2-3</td>
<td>1.42</td>
<td>1.42</td>
<td>1.46</td>
<td>1.42</td>
<td>1.42</td>
</tr>
<tr>
<td>( \alpha ) 3-4</td>
<td>1.20</td>
<td>1.24</td>
<td>1.31</td>
<td>1.23</td>
<td>1.21</td>
</tr>
<tr>
<td>( \alpha ) 4-5</td>
<td>1.12</td>
<td>1.13</td>
<td>1.12</td>
<td>1.12</td>
<td>1.13</td>
</tr>
<tr>
<td>( \alpha ) 5-6</td>
<td>1.19</td>
<td>1.19</td>
<td>1.21</td>
<td>1.21</td>
<td>1.20</td>
</tr>
<tr>
<td>( \alpha ) 6-7</td>
<td>1.08</td>
<td>1.09</td>
<td>1.08</td>
<td>1.08</td>
<td>1.09</td>
</tr>
</tbody>
</table>

The selectivities were also very close for the selected markers, as illustrated in Table 1. Indeed, the change in selectivity was on the maximum equal to 2%. However, when observing in more details the chromatograms of fig. 1, some changes in selectivity could appear. This is evident for the peak of marker 1 (359.06) and the following peak (461.06) which were not fully separated on the Poroshell, but baseline resolved on the Kinetex or Ascentis. The most important change in selectivity was observed for the peaks eluted after the marker number 2 (345.15). This is illustrated in the inset showing the zoom-in of this zone. Using the Kinetex, five peaks (313.05; 345.15; 345.15; 283.04; 345.15) can be identified after the marker 2, while only three of these five peaks (345.15; 283.04; 345.15) were separated on the Poroshell and the Ascentis. This alteration of selectivity between columns could be attributed to the physico-chemical properties of the observed metabolites and the amount of available residual silanols, bonding density and type of endcapping. Thus, even if the 5 phases have C18 bonding, some slight differences can be observed. Last but not least, the peak widths were measured and peak capacity values reported in figure 2A. As shown, the peak capacity varies between 246 and 310 using UV detection and from 187 to 250 with the QqTOF/MS device. It is well known that MS can contribute to additional band broadening because of the ionization source design, optics and electronic signal treatments. This can explain the loss of peak capacity reported in figure 2A and has been discussed elsewhere [42].
In terms of kinetic performance, the Ascentis material seems to be the best one and provide a peak capacity of 310 for a 30 min gradient run, followed by the Acquity (P=282), then Poroshell (P=266), Kinetex (P=260) and finally Hypersil Gold (P=246), which provide the lowest kinetic performance, despite the fact the particle size was small, namely 1.9 \( \mu \)m. To better illustrate the benefits of using sub-3\( \mu \)m core-shell material over columns packed with fully porous sub-2\( \mu \)m particles, the pressure drops were measured at the initial composition of the gradient (2:98 ACN:H2O) and were equal to 344, 376, 391, 607 and 696 bar, for the Ascentis, Kinetex, Poroshell, Hypersil Gold and Acquity, respectively. This result demonstrates that pressure on the Ascentis was more than 2-fold lower than that of the Acquity.

Another example of natural product (Juniperus communis L.) was presented in figure 3. Juniperus communis L. is an evergreen shrub or tree diffused in Europe, Asia and North America. Berries are particularly known for their aromatic properties and they are used for perfumes, flavours or juniper-based spirits, such as gin. They are also used in folk medicine for their diuretic, antiseptic, stomachic and antimicrobial properties [43,44]. Needles are not so much investigated compared to berries, but it could be possible that they present the same secondary active metabolites as the berries, but in different concentrations. In this extract, the complexity of the sample was increased and the number of peaks significantly higher than in Rosemary.
High resolution profiling of complex plant extracts by modern liquid chromatography strategies

Again, there were 6 known markers that were considered for evaluation of selectivity and kinetic performance in table 1 and figure 2B. In this example, the variation of retention was approximately the same as for the previous example. Indeed, the Poroshell and Acquity remain the most retentive phases. However, the changes in selectivity were more drastic compared to figure 1. This is illustrated with the selected markers (see table 1). As example, the variation of selectivity between the marker 4 (539.17) and 5 (537.08) was equal to 22% between the Kinetex and the Hypersil Gold. In addition, the selectivities on the Kinetex phase were quite low for the peaks eluted close to the markers 2 (609.15) and 3 (431.14), compared to the other phases, where a significantly higher number of peaks were chromatographically resolved. Most likely, the important change in selectivity on the juniper compared with the rosemary extract could be explained by the physico-chemical properties of the metabolites contained within the extract and their different interactions with the five stationary phases. For kinetic performance, the column ranking was also slightly modified compared with the previous example (fig. 2A and 2B). With the juniper extract, the peak widths observed on the Ascentis, Kinetex, Hypersil Gold and
Acquity were very close and peak capacity values were comprised between 388 and 408 for these three phases. On the other hand, the performance of the Poroshell was significantly reduced and peak capacity was only equal to 343, using UV detection and down to 270 with QqTOF/MS detection.

The last example of plant extract is the *Gingko Biloba* L.. Corresponding chromatograms were reported in figure 4. In China, preparations of Ginkgo have been used for 5000 years in the treatment of asthma, bronchitis and cardiovascular diseases [45]. The pharmacological activities of ginkgo extracts have been attributed to two different classes of compounds: terpene lactones and flavonols [46, 47]. Terpene lactones are selective antagonists of platelet activating factor, while flavonols have an antioxidant activity. Recently, studies showed efficacy of ginkgo extracts in treatment of cerebrovascular insufficiency, symptom related to Alzheimer disease [45; 47]. Currently, extracts are standardized to contain not less than 22% and not more than 27% flavonoids, calculated as flavonoids glycosides, ad not less than 5% and not more than 7% terpene lactones [47].

Figure 4: Comparison of standardized *Ginkgo Biloba* L. extract analysis on 5 different columns: A) Kinetex C18, B) Poroshell C18, C) Ascentis Express C18, D) Thermo Hypersil Gold C18 and E) Waters Acquity BEH C18. Solvent gradient was kept for 2 minutes at 2% and then increased to 35% of the organic mobile phase in 30 minutes with a flow rate of 0.4 mL min⁻¹. Temperature was 40°C and injected volume 5 µL. MS detection was performed in ESI - mode.
Among the three plant extracts, the Gingko was the most complex one and the number of peaks that could be resolved using the generic gradient of 30 min and the 150 mm highly efficient columns was impressive (several dozens). The variations of selectivity between the different phases using the Gingko extract (up to 8%) were comprised between those observed with rosemary (up to 2%) and juniper (up to 22%). With the Gingko extract, the largest selectivity change was observed between the Poroshell and Kinetex for the markers 3 (325.07) and 4 (609.15). Some interesting observations were made on selectivity between columns. As example, the marker 2 (239.03) co-elute with another peak (325.07) on the Hypersil Gold and Acquity, the resolution was very poor on the Kinetex and Poroshell but they were fully resolved on the Ascentis material. The same remark can be made on several other peaks of the chromatograms, but this is never the same column that gives the best resolution, proving that the selectivity of these different C18 phases was not identical. The peak capacities achieved with the Ginkgo extract were reported in figure 2C. As shown, the Ascentis and Poroshell were the best phases as P values were equal to 293 and 289, respectively. The peak capacity achieved with the Acquity was slightly lower (P=281), while the Hypersil Gold and Kinetex provides P value around 272 and 267, using UV detection. This ranking was surprising as it was again different from that observed with the rosemary and juniper.

Based on the three examples of natural products, the following conclusions can be drawn. i) In terms of retention, the Poroshell and Acquity were systematically the most retentive phases. ii) Regarding selectivities, the most important differences were observed on the juniper extract, probably because of the physico-chemical properties of the metabolites contained within this extract, allowing several types of interaction with the stationary phase (not only a pure hydrophobic mechanism). Among the five tested columns, the Kinetex appears as the phase that provides the most different selectivities for the three tested plant extracts. The selectivity of the four remaining phases was very close, and this is certainly not the main parameter to consider for selecting the most appropriate phase when dealing with high resolution plant profiling. iii) In terms of kinetic performance, the best performance was systematically attained with the Ascentis material, followed quite closely by the Acquity phase. However, the peak capacities attained with three other phases, Kinetex, Poroshell and Hypersil Gold, were significantly poorer. To note, there was always one column which provide notably lower performance for the different extracts and this column was never the same (Hypersil gold for rosemary, Poroshell for juniper and Kinetex for Gingko). This observation proves that the kinetic performance are compound dependent. Thus, it is difficult to make a link between the present study and some other ones reporting kinetic performance, using small neutral analytes or pharmaceuticals as model compounds. iv) The last important feature is the pressure drop generated by these different phases. As discussed, the Ascentis produces the lowest pressure, while the Acquity offers the highest one (around 2-fold higher compared to the Ascentis). As expected, the pressure drop of the columns packed with fully porous sub-2µm particles were significantly higher than that observed with the core-shell sub-3µm particles. However, this result has to be balanced with the mechanical stability of these different phases (600 bar for core-shell material and 1000 bar for UHPLC phases). In conclusion, the Ascentis appears as a very promising material for plant extract analysis. In addition, this phase could be employed on a conventional HPLC system, providing that a temperature of 40-45°C was employed and methanol was avoided as organic modifier (higher viscosity), to limit pressure drop. On the other hand, if a UHPLC system is available, the Acquity phase is also of interest as it can be employed at much higher pressure (up to 1200 bar) and temperature (up to 90°C) than the Ascentis.

Because of the very high resolving power of last generation of TOF/MS device (resolution of >10000 FWHM) and excellent mass accuracy of < 5 ppm, it is sometimes difficult to understand the benefits of increasing the chromatographic resolution and peak capacity. However, when analyzing plant extracts, there are always a significant number of positional isomers and diastereoisomers among the numerous metabolites, that share similar m/z ratio and that cannot
be discriminated even with HRMS device. To illustrate such behaviour, figure 5 shows the extracted mass (m/z 345.15 +/- 0.05 Da) from the rosemary extract shown in figure 1.

Figure 5: Isomer peaks (m/z 435.15) present in the standardized extract of *Rosmarinus officinalis* L. analysed by the five columns: A) Kinetex C18, B) Poroshell C18, C) Ascentis Express C18, D) Thermo Hypersil Gold C18 and E) Waters Acquity BEH C18.

It has been reported that the latter contains three identified positional isomers (i.e. rosmarnol, epirosmanol, episorosmanol) and another one that was not identified [48] but which share the same mass. Except these four peaks, a few additional peaks were also observed in figure 5, but the purpose of this study was not to identify these compounds. However, the chromatograms shown in figure 5 clearly demonstrate that the separation of these different positional isomers was possible, whatever the selected column. The resolution was very large and selectivity was identical between the five phases. This result proves that coupling a high resolution chromatographic method with highly sensitive MS device, could bring benefits, particularly for isomeric compounds.
3.2. Ultra high resolution UHPLC analysis of plant extract

An alternative approach was tested to increase the resolution between the constituents of the plant extract, but without the need to use a high resolution MS platform, as the latter can be unavailable in laboratory. To increase chromatographic resolution, various parameters were investigated, namely the increase of column length, gradient time and mobile phase temperature. For this purpose, the Acquity phase was selected as it provides good results for profiling of plant extract, as demonstrated in §3.1. Compared with core-shell material, this stationary phase packed with fully porous sub-2 μm particles has the additional advantage to be compatible with very high pressure (up to 1000-1200 bar) and temperatures (up to 90°C [49]). The mechanical as well as chemical stability of core-shell material were much more limited (600 bar and 60°C only).

It has been reported that higher peak capacity can be achieved in UHPLC using longer columns packed with sub-2 μm particles and extended gradient times. To maintain similar selectivity between various experiments performed with different column lengths, the gradient steepness should remain identical (i.e. same initial and final gradient composition; similar slope*t₀ values). To meet this requirement, it is recommended to increase the gradient time in direct proportion to the column length. This approach was investigated using the juniper and the gingko extract. For both extracts, three types of experiments were carried out using UV detection. In a first instance, a 150 mm UHPLC column was employed at a flow rate of 0.4 mL/min, generating a backpressure of 753 bar with the initial gradient composition. The corresponding chromatograms with a gradient time of 30 min were reported in figure 6A and 7A.

![Figure 6: Ultra high resolution analysis of methanolic needle extract of Juniperus communis L. obtained by coupling columns together (Waters Acquity BEH C18 150 x 2.1 mm; 1.7 μm) and using high temperature. A) column length, 150 mm; flow rate 0.4 mL min⁻¹; temperature 40°C; injected volume, 5 μL; B) column length, 300 mm; flow rate 0.2 mL min⁻¹; temperature 40°C; injected volume, 10 μL; C) column length, 450 mm; flow rate 0.15 mL min⁻¹; temperature 40°C; injected volume, 15 μL; D) 450 mm; flow rate 0.250 mL min⁻¹; temperature 90°C; injected volume, 15 μL.](image)

Then, two columns of 150 mm were coupled in series to attain a total length of 300 mm. Because of the higher generated backpressure, the flow rate has to be reduced to only 0.2 mL/min, generating a backpressure of 823 bar. To maintain the same gradient steepness, the gradient time was adjusted to 120 min and corresponding chromatograms for juniper and gingko were reported.
in figure 6B and 7B, respectively. Finally, the longest employed UHPLC column was 450 mm and was obtained by coupling in series three columns of 150 mm. Again, because of pressure constraints, the mobile phase flow rate was reduced down to 0.135 mL/min (backpressure of 851 bar) and corresponding chromatograms obtained with a gradient time of 268 min were reported in figure 6C and 7C.

![Figure 7: Ultra high resolution analysis of standardized Ginkgo Biloba L. extract coupling columns together (Waters Acquity BEH C18 150 x 2.1 mm; 1.7 µm) and using high temperature. A) column length, 150 mm; flow rate 0.4 mL min⁻¹; temperature 40°C; injected volume, 5 µL; B) column length, 300 mm; flow rate 0.2 mL min⁻¹; temperature 40°C; injected volume, 10 µL; C) column length, 450 mm; flow rate 0.135 mL min⁻¹; temperature 40°C; injected volume, 15 µL; D) 450 mm; flow rate 0.250 mL min⁻¹; temperature 90°C; injected volume, 15 µL.]

The peak capacities achieved with 150, 300 and 450 mm were equal to 228, 246 and 299 for the Gingko extract, respectively and 277, 297, and 325 for the Juniper extract, respectively. The improvement of peak capacity remains thus limited compared to the increase of analysis time. Indeed, between a gradient time of 30 min and 268 min, the peak capacity was increased by 31% for the Gingko extract and only 17% for the Juniper extract.

Another interesting way to improve peak capacity in the gradient mode is to combine the use of long UHPLC columns (450 mm) with elevated mobile phase temperature. In fact, increasing temperature allows a significant reduction of mobile phase viscosity, allowing to work at higher mobile phase flow rate. In such conditions, it becomes possible to attain very high peak capacity within reasonable timeframe. This is illustrated in fig. 6D and 7D where the separation of Gingko and Juniper were carried out at 90°C, at a flow rate of 0.25 mL/min. In these conditions, the generated backpressure was equal to 819 bar and gradient time was 144 min, to maintain the same gradient steepness. At this flow rate, the column operated closer to the optimum linear velocity and the average or effective retention factor, kₑ was higher than that obtained at 40°C. Thus, the peaks at 90°C appear to be narrower compared to ambient temperature and peak capacity was increased up to 346 for the Gingko and 568 for the Juniper extract. Compared to the experiment performed at 40°C, with a 150 mm column length and gradient time of 30 min,
this corresponds to a gain in peak capacity equal to 51% and 105% for the Gingko and Juniper, respectively, using a gradient time of 144 min at 90°C.

In conclusion, the gain in peak capacity achieved from the increase of column length at ambient temperature is limited. This behaviour is consistent since the flow rate has to be decreased below the optimum of the van Deemter curve, to maintain acceptable backpressure. On the other hand, when combining high temperature (up to 90°C) together with long UHPLC columns, a higher flow rate can be employed, leading to a drastic improvement of chromatographic performance.

3. CONCLUSION
In the present study, advantages and limits of various chromatographic strategies were evaluated to obtain high resolution analyses of complex plant extracts.

Five different 150 x 2.1 mm columns were selected: three columns packed with sub-3 µm core-shell particles and two columns packed with sub-2 µm fully porous particles. Several parameters, such as retention time, selectivity and peak capacity, were compared, using three model plant extracts of increasing complexity. The most retentive phases for all the extracts were the Poroshell and Acquity phases. For the three samples, some differences in selectivity were observed and can vary between 2% for rosemary, and 22% for juniper. The Kinetex phase was systematically the most different phase, in terms of selectivity, among the others. This was attributed to possible differences of silica matrix, bonding density and nature of end-capping.

Kinetic performance was evaluated both with UV and QqTOF/MS detector. As reported in other papers, QqTOF/MS detection provides lower peak capacities than UV one, because of additional band broadening that could occur in the MS device. Nevertheless, with both detectors, Ascentis and Acquity columns offer the best kinetic performance, while the three remaining columns show a significantly lower peak capacity. An additional advantage of the sub-3µm core-shell material is the lower backpressure (in average a factor 2) compared with the fully porous sub-2µm particles. It is well known that QqTOF/MS detector is an important tool to tentatively identify compounds within the sample. However, the quality of the chromatographic separation is also essential to discriminate all isobaric compounds that can be observed within a complex plant extract. As example, in the rosemary extract, all of the five columns were able to baseline separate five isobaric compounds.

As not all of the laboratories specialized in plant analysis have at disposal powerful mass detectors, alternative approaches were tested to increase chromatographic resolution, in combination with a single UV detector. Coupling several columns packed with sub-2µm particles together can be a solution. Indeed, the peak capacity increases progressively with the column length, but the analysis time becomes too long, even for such complex samples. A good alternative is to increase simultaneously the temperature up to 90°C. In fact, analysis time is shortened by 45% compared to analyses carried out at 40°C, with a further increase of peak capacity. However, even with peak capacity higher than 500, the performance of a simple UV detector remains too limited to ensure that no coelutions occur during the analysis.
4. REFERENCES

High resolution profiling of complex plant extracts by modern liquid chromatography strategies

3.2.2 *Populus nigra* L. bud absolute: a case study for a strategy of analysis of natural complex substances

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Analytical and Bioanalytical chemistry

**ABSTRACT**

The new European regulations (e.g. REACH) require that Natural Complex Substances such as essential oils, absolutes, concretes and resinoids are registered. This need implies that the chemical composition of these complex mixtures is characterized as exhaustively as possible in view of defining their toxicological risk. This study proposes a strategy of analysis to be applied to the chemical characterization of poplar absolute as an example of Natural Complex Substances of vegetable origin. In the first part, the proposed strategy is described and the advantages and the limitations related to the combination of conventional analytical techniques such as GC without and with sample derivatization and HPLC are critically discussed. In the second part, the qualitative data obtained with GC and HPLC analysis of poplar bud absolute confirm the sample complexity which mainly consists of phenolic components. Fourteen compounds (i.e. phenolic acids, phenylpropanoids and flavonoids) were then chosen as markers representative of the main classes of components characterizing poplar bud absolute. The marker quantitation carried out by GC-SIM-MS and HPLC-PDA analyses gives similar results confirming the reliability of both techniques. These results demonstrate that conventional analytical techniques can positively and effectively contribute to study the composition of natural complex substances, i.e. matrices for which highly effective separation is necessary, consisting them mainly of isomers or homologous components. The combination of GC and HPLC techniques is ever more necessary for routine quality control when conventional instrumentations are used.

**KEYWORDS:** Natural Complex Substances (NCS); *Populus nigra* L.; high and low volatility compounds; GC derivatization; qualitative analysis; quantitative analysis.
INTRODUCTION

The new regulations, in particular those introduced by the European Community (e.g. REACH), require that Natural Complex Substances (NCS) such as essential oils, absolutes, concretes and resinoids produced or imported in amounts above 1 ton/year in the EC has to be registered together with their toxicological properties and possibly with their environmental impact in order to inform consumers of risk assessment [1]. NCS registration is not easy because of their complexity, since an as exhaustive as possible chemical characterization is mandatory to define its toxicological risk. According to the ISO norms an absolute is "a product with odour obtained from a concrete or a resinoid by extraction with ethanol at room temperature, cooled and filtered to remove the waxes; the ethanol is then removed by distillation" and a concrete (or a resinoid) is "an extract with a characteristic odour from a fresh (or dried) plant raw material by a non-aqueous solvent" (in general hydrocarbons) [2].

In general, an absolute contains hundredths of lipophilic compounds of different volatility that make complex to investigate its chemical composition, as demonstrated by the limited number of articles available [3,4]. An ad hoc strategy to study absolute composition and its biological activity is therefore necessary. This article proposes a possible approach to investigate absolute composition, to define its markers, with the main aim to make it applicable to routine control. This method consists of the combination of a) a non-targeted step that involves an exhaustive analysis to obtain qualitative and semi quantitative composition (mainly expressed as relative percent abundance) enabling the definition of the main classes and/or groups of compounds in the absolute under investigation, followed by b) a targeted step to quantify a number of representative markers selected within the most significant groups in the total extract. Poplar bud (Populus nigra L. -Salicaceae) absolute has here been used to develop and test the proposed strategy. This absolute is widely used in flavor, fragrance and cosmetic industries for its fragrance and for the antioxidative properties due to the presence of phenolic compounds [5,6].

The genus Populus (Salicaceae family) consists of about 30 species [7], growing in southern and central Europe, in central Asia, Siberia and North America, characterized and differentiated by the presence of different flavonoids, phenolic derivatives and terpenoids in particular in the young leaves, buds and bud exudates [8-11]. The species belonging to this genus can chemically be distinguished on the basis of their composition [8-12]. Populus nigra L. buds have been described mainly to contain terpenoids (in particular mono and sesquiterpenoids) and phenolic compounds (phenols, phenolic acids, phenylpropanoids and different subgroups of flavonoids) ([8-10,12-14] and reference cited therein].

This study discusses critically the proposed strategy to analyze a NCS of vegetable origin with conventional techniques in view of its application in routine quality control and the results obtained when it is applied to the poplar bud absolute.

EXPERIMENTAL

Plant Material and chemicals

Poplar absolute from Populus nigra L. buds (lots 221265 and 1772363) was produced by Robertet SA (Grasse, France). Three samples for each lots were analyzed. Pyridine and bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane and benzoic acid, caffeic acid, trans-p-coumaric acid, ferulic acid, isoferulic acid, 3,4-dimethoxy cinnamic acid, trans-cinnamic acid, p-methoxy-cinnamic acid, and 1,1-dimethylallyl caffeate (purity > 98%) were from Sigma-Aldrich (St. Louis MO, USA). Chrysin, galangin, pinocembrin, pinostrobin, tectochrysin (purity > 99%) were from Extrasynthese (Genay, France). HPLC and analytical grade solvents were from Carlo Erba Reagenti (Rodano, Italy).

Vacuum molecular distillation

Poplar bud absolute (300 g) was submitted to vacuum molecular distillation in a KDL4 system (Leybold-Heraeus, Oetikon, Switzerland) for 150 min in Robertet laboratories in Grasse.
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(France). Initial temperature: 60°C, cooling temperature: 50°C. Distillation occurred at 130°C and 1 x 10⁻³ bar under stirring (250 rpm). Molecular distillation provided three fractions of different volatility: 1) a volatile fraction (VF) collected in the first condenser, 2) a distilled fraction (DF) collected in the second condenser, and 3) a residue fraction (RF) containing the components not distilled under the conditions applied. The resulting fractions were preliminarily analyzed by GC–FID, GC-MS, HPLC-PDA, and HPLC-PDA-MS (see previous sections).

Sample and fraction processing
a) **Volatile fraction (VF).** On the basis of the preliminary GC-FID and GC-MS analysis results, the oily yellow VF fraction was submitted to a 1/1 v/v basic extraction with NaHCO₃ (10%) to separate acidic components. Free acids were restored by acidifying the aqueous phase and extracting them with methylene chloride. The acid-free VF was submitted to column chromatography on Silica gel with petroleum ether/ethyl acetate varying from 100/0 to 70/30.

b) **Distilled fraction (DF).** The light yellow gummy DF sample was dissolved in ethyl acetate and submitted to the same treatments as for VF fraction (i.e. deacidification), and column chromatography under the same conditions.

c) **Fraction and subfraction derivatization.** Pyridine (80 μl) and bis(trimethylsilyl)-trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (120 μl) were added to 2 mg of each fraction (VF, DF and RF and related subfractions) and heated at 60°C for 30 min to derivatize hydroxylated and carboxylic components to the corresponding trimethylsilyl derivatives (TMS).

**GC-FID and GC-MS analysis**
GC analyses were carried out on a Shimadzu 2010 GC-FID system and a Shimadzu QP2010 plus GC-MS system, both provided with an AOC-20i automatic injector, and with Shimadzu GC Solution 2.53SU1 software and Shimadzu GCMS Solution 2.51 software, respectively (Shimadzu, Milan, Italy).

GC-FID and GC-MS analyses were carried out on a Mega 5 column (95% polydimethyl-siloxane, 5% phenyl) 25 m x 0.25 mm d.c. x 0.25 μm d.f. from MEGA (Milano – Italy).

GC-FID and GC-MS conditions: injection mode: split; split ratio: 1:20; injection volume: 1 μl. Temperatures: injector: 250°C, FID: detector: 280°C, MS: transfer line: 280°C; ion source: 200°C; carrier gas: He, initial flow-rate 1.0 ml/min in constant linear velocity mode. Temperature programme: from 50°C (1 min) to 280°C (10 min) at 3°C/min. The MS operated in electron impact ionization mode (EI) at 70 eV, at a scan rate of 1111 u/s with a mass range of 35–750 m/z, suitable to cover the full fragmentation pattern of all analytes investigated in full scan mode; an interval time of 0.2 s was applied in SIM mode to three diagnostic ions for each standard component.

Absolute volatile components were identified by comparison of both their linear retention indices (Iₜₜₕ), calculated versus a C₉-C₅₆, C₅₈, C₃₂, C₃₄ hydrocarbon mixture, and their mass spectra to those of authentic samples, or data from home-made or from commercial libraries or reported in the literature [12, 15-18]

Quantitative GC-SIM-MS analysis. Suitable amounts of caffeic acid, trans-p-coumaric acid, ferulic acid, isoflavonolic acid, benzoic acid, 1,4-dimethoxy-cinnamic acid, trans-cinnamic acid, p-methoxy-cinnamic acid, 1,1 dimethylallyll caffeate, chrysin, galangin, pinocembrin, pinostrobin, tectochrysin were diluted with acetone to obtain 5 different concentrations in the range of 0.05 to 0.5 μg/μl for each marker. 1 ml of standard solution for each concentration was evaporated to dryness and derivatized to the corresponding trimethylsilyl derivatives (TMS) as reported above. Each concentration was derivatized in triplicate. Calibration curves were built by analyzing the resulting TMS-derivatized standard solutions three times by GC-MS in SIM mode under the conditions reported above.

HPLC-PDA-MS analysis
DF and RF were analyzed by a Shimadzu LC-MS 2010EV system equipped with a photodiode detector SPD-M20A (Shimadzu, Dusseldorf Germany) in series to a single quadrupole MS
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A system provided with orthogonal atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) sources. An Ascentis Express C18 column (150 x 4.6 mm I.D., 2.7 μm), (Supelco, Bellefonte, PA) was used. Analysis conditions were: temperature: 40°C; mobile phase: eluent A: water/0.1% formic acid; eluent B: methanol/0.1% formic acid; mobile phase gradient was as follows: 40% B for 10 min, 40-48% B in 10 min, 48-60% B in 10 min, 60% for 10 min, 60-90% B in 40 min and 90% B for 10 min. Injection volume: 5 μL, flow rate: 0.4 mL/min. UV spectra were acquired in the 210-450 nm wavelength range and the resulting chromatograms were integrated at different wavelengths in function of the UV absorption maxima of each component. MS operative conditions: ESI temperature: 200°C; nebulizer gas flow rate: 1.5 mL/min; curve desolvation line (CDL) temperature: 250°C. Mass spectra were acquired both in positive and in negative full-scan mode in the range of 100-700 m/z, with a scan range of 1000 u/s.

Quantitative HPLC-PDA analysis. Suitable amounts of caffeic, trans-p-coumaric, ferulic, isoferulic, benzoic, dimethyl caffeic, trans-cinnamic and p-methoxy-cinnamic acid, caffeic acid 1,1-dimethyl allyl ester, chrysin, galangin, pinocembrin, pinostrobin, tectochrysin were dissolved in methanol to obtain concentrations from 1 to 50 μg/mL of each marker. Calibration curves were built by analyzing the resulting standard solutions three times by HPLC-PDA.

RESULTS AND DISCUSSION
This section consists of two main parts: 1) a critical discussion of the strategy to adopt for NCS analysis; 2) the results obtained by its application to the poplar bud absolute.

Strategy of analysis
As already mentioned, this strategy combines a non-targeted step to define as exhaustively as possible qualitative composition and to measure the indicative percentage abundance of the main groups of compounds in the absolute under investigation, and a targeted step to select and quantify a number of representative markers suitable to characterize it. Moreover, in view of the use of this strategy in quality control, techniques conventionally available in routine laboratory are here adopted. The strategy of analysis of course depends on the complexity of the specific absolute, although, in general, because of the number of components that accounts for some hundredths, the original sample has to be “simplified” through a preliminary fractionation to meet the aim to study its composition exhaustively. The process to obtain absolutes excludes both highly apolar and polar compounds (e.g. waxes, fats, glycosides etc.) while it does not discriminate between high and low volatility compounds. These sample characteristics mean that, in general, an absolute is not analyzable in a single run as such and/or with a single chromatographic technique (e.g. HPLC or GC). One of the approaches that leaves out of the nature of the absolute components is a fractionation on the basis of their volatility through a vacuum or molecular distillation, the vacuum being necessary to avoid high temperatures inducing artefact formation and limiting component thermal degradation. This operation should provide fractions of homogeneous volatility and therefore analyzable in toto with the most appropriate technique. Further fractionation based on acid/basic extractions or on polarity by SPE, column or flash chromatography, preparative LC can afterward be carried out to simplify ever more the composition of the fractions from distillation or to isolate unknown markers (where present) and elucidate their structures by spectroscopic techniques (e.g. NMR). Molecular distillation of the investigated poplar bud absolute has produced three fractions; as reported below it met the aim to simplify sample composition, since the first fraction consisted of volatiles (volatile fraction, VF) mainly sesquiterpenoids, the second one of medium volatility components (distilled fraction, DF) containing above all phenolic derivatives and a residue containing high boiling compounds (residue fraction, RF).
Several studies have demonstrated how important is to combine results from GC and HPLC in the quali-quantitative analyses of natural complex mixtures as an approach to overcome the intrinsic limitations of these two techniques [19-22]. However, the conventional analysis of an absolute such as that of poplar buds is conditioned by some objective limits of these two separation techniques. GC is limited in the analysis of high volatility compounds, although its use can be extended to medium-high boiling and medium polarity compounds by modifying their volatility through suitable derivatisation reactions. The role of GC-MS including derivatization in plant metabolite profiling was critically discussed by Halket et al. [23] and Fiehn [24]. More recently, Isidorov and Szczepaniak [18] reported the retention indices of 389 biologically and environmentally important organic compounds determined with moderately polar columns (polydimethylsiloxane-5%-phenyl) most of them derivatized to the corresponding trimethylsilyl derivatives (TMS) with pyridine and N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% of trimethylchlorosilane and evaluated $I_T^S$ reliability both of underivatized and of the corresponding derivatized analytes and the factors influencing their reproducibility. Analyte derivatisation offers: 1) an FID response to analytes only depending on their amounts at least within a homogeneous group of compounds, 2) the possibility to apply $I_T^S$ to locate (and identify) analytes in the total chromatogram, 3) mass spectra with diagnostic and reproducible fragmentation patterns, 4) improvement of the peak shape of components interacting with the stationary phase producing leading or tailing peak distortion (in this case e.g. benzoic acid), thus increasing the reliability of quantitative data. On the other hand, derivatization of low volatility components requires: a) a correct application of percent normalization by using an internal standard and FID response factors [25], b) availability of reference standards for a correct identification and quantitation, c) careful tuning of derivatization conditions to achieve high yield and avoid multiple products due to incomplete derivatization of molecules containing hydroxyls with a different reactivity, that can make the chromatogram ever more complex, and d) possibility of alteration of the gas chromatographic (injector, liner activity, column efficiency) and MS performance due to possible decomposition of reagents and derivatized analyte(s). Lastly, GC-MS can exploit commercially, literature and/or in-house available linear retention index ($I_T$) collections [15, 18, 26-28] interactively or not combined to mass spectral libraries as a tool for component identification, unlike what happens in HPLC-MS (or UV).

HPLC also shows some limitations in the analysis of highly complex mixtures although its most recent advances (Ultra High Pressure LC (UHPLC) and the introduction of evoluted IT or TOF analyzers and hybrid systems ([29,30] and references cited therein)) have dramatically increased its performance. In particular a) HPLC-UV is unable to detect compounds without chromophoric groups; moreover, its response depends on analyte molar absorbivity that is structure-specific, thus altering the relative ratios between the sample components and making impossible to establish a correct ratio between peak abundances and, as a consequence, a quantitation (or at least semi-quantitation) without reference standards, and b) HPLC-MS gives responses that are again influenced by i) the analyte structure, whose fragmentation also depends on the adopted HPLC-MS interface and ion source, ii) the analyte ionization rate that can vary depending on structures, iii) the nature of the analyzer, and iv) the limitations in mobile phase composition and flow-rate that can affect HPLC separation flexibility.

The analysis of natural complex substances and in particular of poplar bud absolute implies the fundamental complementary use of all discussed techniques since: i) GC-FID or MS without derivatisation is always necessary not only to provide with a characterization of volatiles and their rate but also as a reference GC profile to be compared to that of the derivatized sample as here shown for sesquiterpene hydrocarbon identification and flavonoid quantitation (see next paragraphs), ii) GC-MS with derivatisation makes analyzable by GC poorly or not volatilizable or medium-high polarity compounds contributing to their identification and making possible their quantitation, and iii) HPLC-UV and MS are indispensable for identification and quantitation of non-volatile compounds. These techniques could indifferently be used for quantitation of selected markers provided that standards are available and the investigated peaks
are correctly eluted and separated, thus making possible a cross control of the results. In addition, GC-FID can provide reliable semi-quantitation (% normalization) and peak relative abundance provided that an internal standard and average FID response factors are available. GC-FID without derivatization is the preferred technique for semi-quantitation preliminary but important indispensable step for sample comparison.

**Qualitative and Quantitative analyses of poplar bud absolute**
This study aims to characterize the chemical composition of the absolute obtained from *Populus nigra* L buds. Fig. 1 reports the GC-MS profiles as such and after derivatization of the total absolute and Fig. 2 the corresponding HPLC-PDA profile.

![Figure 1: Poplar absolute GC-MS profiles as such (A) and after derivatization (B). Peak numbers are referred to those reported in Table 1.](image1.png)

![Figure 2: HPLC-PDA profile of poplar absolute. The chromatogram has been acquired at 280 nm. Peak numbers are referred to those reported in Table 1.](image2.png)

Poplar bud absolute was first submitted to molecular distillation from which three fractions of homogeneous volatility were obtained: VF (representing about 4% of the total absolute), DF (about 20%) and RF (about 75%). Fig S1 and S2 (supplementary data files) show the...
effectiveness of the molecular distillation. The repeatability resulting from molecular distillation of the six samples from the two lots of poplar bud absolute was good giving RSD% of 10% for VF, 7% for DF and 4% for RF. Each fraction was then deacidified, and submitted to a further fractionation by silica column chromatography in order to simplify furthermore the chemical profiles and to facilitate component location and identification. The three main fractions and those resulting from the following treatments were all analyzed by GC-FID-MS as such and/or after derivatization with BSTFA and by HPLC-PDA-MS.

**GC-MS analysis**

A total of 215 and 170 peaks were respectively counted in GC-FID-MS absolute profiles with and without derivatization. Tables 1a and 1b report the list of the identified components in the total absolute and in the three fractions obtained from molecular distillation analysed by GC-FID-MS with (1a) and without (1b) derivatization.
Table 1A: Poplar absolute composition obtained through GC-FID-MS analysis after derivatization

<table>
<thead>
<tr>
<th>ID</th>
<th>Compounds</th>
<th>Fraction</th>
<th>( N^0 ) TMS groups</th>
<th>( I^2_{\text{exp}} )</th>
<th>( I^2_{\text{from literature}} )</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2-Methyl-2-butenenoic acid</td>
<td>VF</td>
<td>1</td>
<td>1020</td>
<td>1015&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.6</td>
</tr>
<tr>
<td>2</td>
<td>Hexanoic acid</td>
<td>VF</td>
<td>1</td>
<td>1081</td>
<td>1077&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.03</td>
</tr>
<tr>
<td>3</td>
<td>Linalool</td>
<td>VF</td>
<td>0</td>
<td>1102</td>
<td>1097&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.1</td>
</tr>
<tr>
<td>4</td>
<td>Camphor</td>
<td>VF</td>
<td>0</td>
<td>1146</td>
<td>1146&lt;sup&gt;a&lt;/sup&gt;</td>
<td>nd</td>
</tr>
<tr>
<td>5</td>
<td>Benzyl alcohol</td>
<td>VF</td>
<td>1</td>
<td>1160</td>
<td>1153&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.3</td>
</tr>
<tr>
<td>6</td>
<td>Benzyl acetate</td>
<td>VF</td>
<td>0</td>
<td>1168</td>
<td>1162&lt;sup&gt;a&lt;/sup&gt;</td>
<td>nd</td>
</tr>
<tr>
<td>7</td>
<td>Ethyl benzoate</td>
<td>VF</td>
<td>0</td>
<td>1173</td>
<td>1163&lt;sup&gt;a&lt;/sup&gt;</td>
<td>nd</td>
</tr>
<tr>
<td>8</td>
<td>Phenethyl alcohol</td>
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Both tables report the components detected by GC-FID-MS analysis together with their \( I^F_S \). The components listed in table 1a and 1b without a percent value were not found in the undervatized and/or derivatized total chromatograms because present in very low amounts and only detected in the absolute fractions. In addition, in table 1a, the number of TMS-derivatized groups is reported for each derivatizable component. The supplementary files S3 and S4 report the mass spectra of the main unidentified components (> 0.2%) in the GC-FID-MS profiles with and without the derivatization procedure together with their \( I^F \). The identification was confirmed with the co-injection of authentic standards (when available) after derivatization. VF was mainly characterized by a mixture of terpenoids, phenolic acids, alcohols and esters, fatty acids and related esters with \( I^F_S \) calculated vs an homologous series of hydrocarbons up to 2200 on polydimethylsiloxane, 5% phenyl as GC stationary phase, and molecular weights (m.w.) up to around 300 (ethyl linoleate m.w. 308 (55)); DF mainly consisted of flavonoid aglycones, cinnamic acid esters, phenolic acids with \( I^F_S \) up to 3200 and a M.W. up to about 450 (triacontane, \( C_{32}H_{66} \)); RF mainly contained phenolic compounds. VF fraction contains several sesquiterpene hydrocarbons (most of them present in very low amounts or traces), sesquiterpenoids (α- and β-eudesmol), phenols and phenolic acids and derivatives (the main one of them being benzoic acid (9), in the total absolute) together with glycerol (14) and catechol (15), fatty acids and related esters (hexadecanoic acid (50) and ethyl hexadecanoate (palmitate) (48). Sesquiterpene hydrocarbons always present as minor or trace components were mainly
identified with the underivatized GC analysis being not detectable in the absolute derivatized GC profile and difficult to detect in the derivatized VF fraction because their spectra were not sufficiently significant due to the higher noise. DF fraction mainly consists of homologous series of cinnamic acids (p-coumaric (47) and p-methoxy-cinnamic (46) acids) and their esters (3-methyl-2-butenyl-p-coumarate (59), dimethyl allyl caffeoate (70), benzyl-p-coumarate (72), benzyl caffeoate (81)) and a series of flavonoids and derivatives (pinostrobin (67,71), pinocembrin (73), pinobanksin (74), chrisyn (75, 82), galangin (80,84) in different abundances) all without hydroxyls on the B-ring. Flavonoids deriving from p-coumaroyl-CoA/naringenin chalcone and caffeoyl-CoA/eriodictyol chalcone biosynthetic pathways (i.e. with hydroxyls in ring B) already identified in P. nigra [8, 31-33] were not found most probably because they are not extracted by the apolar solvent in absolute preparation due to their higher polarity. RF fractions had a composition quite similar to that of DF fractions with an higher amount of the less volatile components. Most of the components identified in the absolute have been previously identified in P. nigra bud exudates [8-10,12-14,31,32,34-35] by different methods of extraction followed by GC-MS analyses with or without derivatization. Some components are only tentatively identified because of both the lack of pure standards and/or of TMS of the TMS derivatives that limits their identification to the comparison to mass spectra available from libraries or from the literature. Percent normalization is here applied as a preliminary indication of abundance of identified and unidentified peaks. Unfortunately some phenolic acids present very bad peak shape (e.g. benzoic acid (9)) or are in very low amounts (e.g. p-coumaric acid (47)) when analyzed without derivatization that make difficult a correct peak area integration thus affecting the resulting percentages. GC-FID with derivatization produces phenolic acid peaks easy to integrate but interfering with flavonoid measurement (see below). 91 components were characterized by GC-MS with derivatization and 52 by GC-MS without derivatization. On the basis of component relative % abundance, the absolute consists of about 26% of flavonoids, about 22% of phenolic acids and about 14% of phenolic esters. Terpenoids, aromatic alcohols, and fatty acids and esters have to be considered as minor groups each one representing about 2% of the total absolute.
Table 1B: Poplar absolute composition obtained through GC-FID-MS analysis without derivatisation.

<table>
<thead>
<tr>
<th>ID</th>
<th>Compounds</th>
<th>Fraction</th>
<th>$t_r$ FID-MS</th>
<th>$t_r$ exp</th>
<th>$t_r$ from literature</th>
<th>%</th>
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<tbody>
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<td>1</td>
<td>2-Methyl-2-butenolic acid</td>
<td>VF</td>
<td>970</td>
<td>970</td>
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<tr>
<td>2</td>
<td>Benzyl alcohol</td>
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<td>1039</td>
<td>1032&lt;sup&gt;d&lt;/sup&gt;</td>
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</tr>
<tr>
<td>3</td>
<td>Linoleal</td>
<td>VF/DF</td>
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<tr>
<td>4</td>
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<td>VF</td>
<td>1115</td>
<td>1107&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>1162&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
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<td>37</td>
<td>Ethyl hexadecanoate</td>
<td>VF/DF</td>
<td>1993</td>
<td>1993&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>38</td>
<td>3-Methyl-3-butenyl-p-coumarate</td>
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<td>2116</td>
<td>2110&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>DF</td>
<td>2135</td>
<td>2135&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>2141&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>41</td>
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<td>2154&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>2171</td>
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<tr>
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<td>Pinostrobin</td>
<td>DF/RF</td>
<td>2391</td>
<td>2386&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
<td>48</td>
<td>Pinocembrin</td>
<td>DF/RF</td>
<td>2478</td>
<td>2480&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
<td>49</td>
<td>Tectochrysin + coelut</td>
<td>DF/RF</td>
<td>2576</td>
<td>2576&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
<td>50</td>
<td>Chrysos + coelut</td>
<td>DF/RF</td>
<td>2669</td>
<td>2666&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
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<td>Galangin</td>
<td>DF/RF</td>
<td>2719</td>
<td>2718&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>52</td>
<td>Pinobanksin-5-methylether (t)</td>
<td>DF/RF</td>
<td>2756</td>
<td>2756&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.6</td>
<td></td>
</tr>
</tbody>
</table>

ID number is related to the compounds of Table 1A.
Compounds whose reference standards are not available are in Italics;
marker compounds used for the true quantitation are in bold;
n.d.: compounds not detectable in the total chromatogram but present as a trace in the fractions;
(t) tentative in agreement with the LC data
<sup>c</sup>: from ref [15];<sup>d</sup>: from ref [18]

**HPLC-PDA-MS analysis**

Poplar bud absolute, DF and RF and their sub-fractions were in parallel analyzed by HPLC-PDA-ESI-qMS. Table 2 reports the list of the componentes identified by HPLC-PDA-ESI-qMS in the total absolute, DF and RF together with their UV absorption maxima and MS data, while Fig. 2 reports the HPLC-PDA chromatogram of the total absolute.
Table 2: Poplar absolute composition obtained through HPLC-PDA-esiMS analysis.

<table>
<thead>
<tr>
<th>ID</th>
<th>Compounds</th>
<th>RT (min)</th>
<th>UV λ max (nm)</th>
<th>M.W.</th>
<th>ESI scan +</th>
<th>ESI scan -</th>
</tr>
</thead>
<tbody>
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<td>53</td>
<td>Caffeic acid</td>
<td>5.32</td>
<td>325</td>
<td>180</td>
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<td>p-Coumaric acid</td>
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<td>164</td>
<td>147</td>
<td>163, 119</td>
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<td>Pinobanksin-3-butanolate</td>
<td>65.366</td>
<td>293</td>
<td>342</td>
<td>343</td>
<td>341</td>
</tr>
<tr>
<td>87</td>
<td>Cinnamyl p-coumarate</td>
<td>65.728</td>
<td>313</td>
<td>280</td>
<td>303 [M+Na]+</td>
<td>279</td>
</tr>
<tr>
<td>79</td>
<td>Tectochrysin</td>
<td>66.816</td>
<td>267/306</td>
<td>268</td>
<td>269</td>
<td>267</td>
</tr>
<tr>
<td>89</td>
<td>Pinobanksin-3-pentanoate</td>
<td>71.665</td>
<td>293</td>
<td>356</td>
<td>357</td>
<td>355</td>
</tr>
<tr>
<td>85</td>
<td>Pinobanksin-3-hexanoate</td>
<td>76.48</td>
<td>292</td>
<td>370</td>
<td>371</td>
<td>369</td>
</tr>
<tr>
<td>43</td>
<td>Cinnamyl p-methoxy-cinnamate</td>
<td>79.73</td>
<td>278</td>
<td>294</td>
<td>295</td>
<td>293</td>
</tr>
</tbody>
</table>

ID number is related to the compounds of Table 1A
Marker compunds are in bold
*: confirmed with ref. [35,37]
*: tentative on the basis of UV and MS data and coherence with the GC-MS analysis.

VF fraction was not analyzed by HPLC because the volatility of its components made GC the technique of choice for its analysis. The components were located in the chromatograms in function of their elution order thanks to the available standards, the analysis of some of the DF and RF sub-fractions also analyzed by GC-MS and the data reported in literature [36,37]. Mass spectra were acquired in positive and negative ESI ionization full scan, in general providing a protonated [MH]+ or a deprotonated [M–H]– molecular ions and, in positive mode, a molecular ion adduct [M + Na]+. The molecular mass of an unknown component was retained only when both [MH]+ and [M–H]– ions were detected. LC-MS data are here fundamental not only for component characterization and location in the chromatogram but also as a complement to confirm GC-MS identification. HPLC results confirmed that *Populus nigra* absolute mainly consists of three groups of phenolic compounds (phenolic acids, phenolic esters and flavonoids) as already shown by GC analysis. 30 components were characterized both by HPLC-PDA-MS analysis, two of them not identified by GC-MS with or without derivatization.

Quantitation of the marker components of poplar absolute
On the basis of the above results showing the prominence of the three phenolic fractions and of the availability of standards, fourteen phenolic compounds were selected as markers to characterize the poplar bud absolute and quantified via an external calibration by both GC-SIM-MS and HPLC-PDA to validate the data by two independent chromatographic methodologies.
### Table 3: Poplar absolute markers and parameters adopted for their quantitation.

<table>
<thead>
<tr>
<th>N°</th>
<th>Compounds</th>
<th>(I^s)</th>
<th>Target ions</th>
<th>(r^2)</th>
<th>Linearity (\mu g/mL)</th>
<th>LOD (\mu g/mL)</th>
<th>LOQ (\mu g/mL)</th>
<th>R.t. (min)</th>
<th>(\lambda_{max})</th>
<th>(r^2)</th>
<th>Linearity (\mu g/mL)</th>
<th>LOD (\mu g/mL)</th>
<th>LOQ (\mu g/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>Benzoic acid</td>
<td>1251</td>
<td>179, 105, 77</td>
<td>0.999</td>
<td>0.05-0.5</td>
<td>0.008</td>
<td>0.022</td>
<td>13.51</td>
<td>230</td>
<td>0.996</td>
<td>1-50</td>
<td>0.005</td>
<td>0.018</td>
</tr>
<tr>
<td>36</td>
<td>Cinnamic acid</td>
<td>1544</td>
<td>205, 131, 161</td>
<td>0.997</td>
<td>0.05-0.5</td>
<td>0.028</td>
<td>0.092</td>
<td>22.23</td>
<td>277</td>
<td>0.999</td>
<td>1-50</td>
<td>0.006</td>
<td>0.019</td>
</tr>
<tr>
<td>46</td>
<td>(p)-Methoxycinnamic acid</td>
<td>1834</td>
<td>161, 235, 250</td>
<td>0.991</td>
<td>0.05-0.5</td>
<td>0.039</td>
<td>0.129</td>
<td>23.21</td>
<td>310</td>
<td>0.994</td>
<td>1-50</td>
<td>0.008</td>
<td>0.028</td>
</tr>
<tr>
<td>47</td>
<td>(p)-Coumaric acid</td>
<td>1951</td>
<td>73, 219, 308</td>
<td>0.997</td>
<td>0.05-0.5</td>
<td>0.004</td>
<td>0.014</td>
<td>9.22</td>
<td>310</td>
<td>0.998</td>
<td>1-50</td>
<td>0.001</td>
<td>0.002</td>
</tr>
<tr>
<td>49</td>
<td>3,4-Dimethoxycinnamic acid</td>
<td>2037</td>
<td>191, 265, 280</td>
<td>0.991</td>
<td>0.05-0.5</td>
<td>0.111</td>
<td>0.369</td>
<td>17.55</td>
<td>325</td>
<td>0.999</td>
<td>1-50</td>
<td>0.007</td>
<td>0.023</td>
</tr>
<tr>
<td>51</td>
<td>Isoferulic acid</td>
<td>2091</td>
<td>73, 338, 308</td>
<td>0.995</td>
<td>0.05-0.5</td>
<td>0.006</td>
<td>0.022</td>
<td>12.27</td>
<td>325</td>
<td>0.999</td>
<td>1-50</td>
<td>0.002</td>
<td>0.008</td>
</tr>
<tr>
<td>52</td>
<td>Ferulic acid</td>
<td>2105</td>
<td>73, 338, 249</td>
<td>0.991</td>
<td>0.05-0.5</td>
<td>0.007</td>
<td>0.022</td>
<td>10.82</td>
<td>325</td>
<td>0.998</td>
<td>1-50</td>
<td>0.003</td>
<td>0.008</td>
</tr>
<tr>
<td>53</td>
<td>Caffeic acid</td>
<td>2157</td>
<td>73, 219, 396</td>
<td>0.995</td>
<td>0.05-0.5</td>
<td>0.011</td>
<td>0.038</td>
<td>6.18</td>
<td>325</td>
<td>0.999</td>
<td>1-50</td>
<td>0.002</td>
<td>0.008</td>
</tr>
<tr>
<td>70</td>
<td>1,1-Dimethylallyl caffeate</td>
<td>2427</td>
<td>73, 219, 292</td>
<td>0.990</td>
<td>0.05-0.5</td>
<td>0.019</td>
<td>0.064</td>
<td>45.34</td>
<td>325</td>
<td>0.999</td>
<td>1-50</td>
<td>0.025</td>
<td>0.083</td>
</tr>
<tr>
<td>71</td>
<td>Pinostrobin</td>
<td>2514</td>
<td>327, 73, 238</td>
<td>0.983</td>
<td>0.1-0.5</td>
<td>0.424</td>
<td>1.415</td>
<td>63.19</td>
<td>289</td>
<td>0.997</td>
<td>1-50</td>
<td>0.029</td>
<td>0.098</td>
</tr>
<tr>
<td>73</td>
<td>Pinocembrin</td>
<td>2551</td>
<td>73, 385, 296</td>
<td>0.995</td>
<td>0.1-0.5</td>
<td>0.007</td>
<td>0.022</td>
<td>43.34</td>
<td>290</td>
<td>0.993</td>
<td>1-50</td>
<td>0.014</td>
<td>0.047</td>
</tr>
<tr>
<td>79</td>
<td>Tectochrysin</td>
<td>2704</td>
<td>325, 155, 282</td>
<td>0.995</td>
<td>0.3-1.00</td>
<td>1.252</td>
<td>4.172</td>
<td>65.49</td>
<td>267</td>
<td>0.998</td>
<td>1-50</td>
<td>0.008</td>
<td>0.027</td>
</tr>
<tr>
<td>82</td>
<td>Chrysin</td>
<td>2746</td>
<td>383, 73, 311</td>
<td>0.991</td>
<td>0.25-0.5</td>
<td>0.028</td>
<td>0.092</td>
<td>46.48</td>
<td>267</td>
<td>0.997</td>
<td>1-50</td>
<td>0.015</td>
<td>0.051</td>
</tr>
<tr>
<td>84</td>
<td>Galangin</td>
<td>2770</td>
<td>571, 73, 399</td>
<td>0.996</td>
<td>0.05-0.5</td>
<td>0.012</td>
<td>0.040</td>
<td>50.11</td>
<td>265</td>
<td>0.995</td>
<td>1-50</td>
<td>0.063</td>
<td>0.210</td>
</tr>
</tbody>
</table>
These compounds are listed in Tables 3 and 4 and were chosen also because the absolute qualitative composition is influenced by the procedure adopted for its preparation that limits the flavonoids to those belonging to the subgroups of flavanones, flavones and dihydroflavonols deriving from cinnamoyl-CoA and pinostrobin chalcone without substituents in B ring. Table 4 reports the diagnostic ions selected for GC-SIM-MS quantitation, absorption maxima wavelengths (λ max) adopted for HPLC-PDA quantitation, together with the calibration ranges, correlation coefficients (r²) and detection and quantitation limits (LOD and LOQ) of each marker.

Table 4: Data comparison of poplar bud absolute markers after true quantitation by derivatization-GC-MS and HPLC-PDA.

<table>
<thead>
<tr>
<th>No</th>
<th>Compounds</th>
<th>Der-GC-MS mg/100mg (%)</th>
<th>HPLC-PDA mg/100mg (%)</th>
<th>RSD%</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>Benzoic acid</td>
<td>1.24</td>
<td>1.28</td>
<td>1.2</td>
</tr>
<tr>
<td>36</td>
<td>Cinnamic acid</td>
<td>0.23</td>
<td>0.21</td>
<td>0.4</td>
</tr>
<tr>
<td>46</td>
<td>p-Methoxycinnamic acid</td>
<td>1.30</td>
<td>1.3</td>
<td>0.0</td>
</tr>
<tr>
<td>47</td>
<td>p-Coumaric acid</td>
<td>1.92</td>
<td>1.8</td>
<td>4.6</td>
</tr>
<tr>
<td>49</td>
<td>3,4-Dimethoxycinnamic acid</td>
<td>1.30</td>
<td>1.37</td>
<td>13.5</td>
</tr>
<tr>
<td>51</td>
<td>Isoflavonoid</td>
<td>0.99</td>
<td>0.93</td>
<td>4.1</td>
</tr>
<tr>
<td>52</td>
<td>Ferulic acid</td>
<td>0.42</td>
<td>0.32</td>
<td>19.0</td>
</tr>
<tr>
<td>53</td>
<td>Caffeic acid</td>
<td>0.44</td>
<td>0.34</td>
<td>18.9</td>
</tr>
<tr>
<td>70</td>
<td>1,1-Dimethylallyl caffeate</td>
<td>0.92</td>
<td>0.99</td>
<td>5.1</td>
</tr>
<tr>
<td>71</td>
<td>Pinostrobin</td>
<td>3.48</td>
<td>3.12</td>
<td>29.6</td>
</tr>
<tr>
<td>73</td>
<td>Pinocembrin</td>
<td>2.79</td>
<td>2.64</td>
<td>18.8</td>
</tr>
<tr>
<td>79</td>
<td>Tectochrysin</td>
<td>1.87</td>
<td>1.77</td>
<td>11.6</td>
</tr>
<tr>
<td>82</td>
<td>Chrysin</td>
<td>2.78</td>
<td>4.15</td>
<td>28.1</td>
</tr>
<tr>
<td>84</td>
<td>Galangin</td>
<td>2.24</td>
<td>2.89</td>
<td>17.9</td>
</tr>
</tbody>
</table>

Table 4 compares the amounts of the 14 markers in the poplar bud absolute under investigation obtained with GC-SIM-MS after derivatization (n = 3) and with HPLC-PDA (n = 3) expressed as mg/100mg of total absolute together with the RSD% between the results. The quantitative results of the main components using the two different methods are comparable for most markers (RSD% < 20) and emphasizes the reliability of both techniques in quantifying the markers of this complex matrix. RSD% values of some flavonoids (in particular pinostrobin, 29.6%, and chrysin, 28.1%) when analyzed by GC-SIM-MS with derivatization and HPLC-PDA above 20% can either be due to possible coelution with other components with both derivatization GC-MS and HPLC-PDA, or to an incomplete derivatization of them (table 1a). The latter explanation was also confirmed by the relatively narrow range of linearity obtained for these compounds, by the detection of partially silylated derivatives and by the fact that their HPLC-PDA-UV quantitative data well agreed with those obtained with GC-SIM-MS without derivatization as it is shown by RSD% that are always below 20% (pinostrobin (6.1%) and chrysin (18.3%), although these compounds present free hydroxyl(s) in the ring A of the flavonoidic skeleton. The quantitation of components for which standards are not available in HPLC-PDA analysis, was carried out by adopting the calibration curves of available standards belonging to the same chemical groups and with the same chromophores and UV absorption maxima, e.g. the calibration curves of ferulic and caffeic acid can be used to quantify ferulic and caffeic esters. The quantitative analysis of the 14 markers and their derivatives selected with the above approximation afforded to quantify 45% by weight of the absolute.
CONCLUSIONS
The proposed strategy has here been shown to be able to characterize the poplar bud absolute because it affords to characterize about one hundred components, the main ones of them belonging to the phenolic groups that account for about 45% of the whole absolute, and to define and quantify fourteen markers representative of about 20% of the sample. These results show how non-targeted and targeted methods can successfully be combined when a complex mixture of vegetable origin has to be studied, and how GC-MS with and without derivatization, HPLC-PDA, and HPLC-MS play a fundamental complementary role in the characterization of complex mixtures consisting of components of different volatility and polarity. As already stressed by other authors [18,36], all techniques equally and sinergically concur to the highly heterogeneous and natural complex mixture characterization, in particular when studies are carried out with conventional instrumentation (GC-FID-MS and HPLC-PDA-qMS). This approach will probably be even more effective by the routine adoption of the recent and more powerful HPLC-MS and GC-MS systems based on high resolution IT or TOF analyzers as such or in a hybrid combination (q-TOF or IT-TOF), that increase the capability of component identification since they can provide molecular formulae and enable to propose hypothesis of structures through dedicated software [38]. However, although very powerful, these instrumentations too are limited by the absolute composition, which mainly consists of isomers or homologous components. These results furthermore confirm the importance of derivatization that makes possible to use GC in the analysis of components poorly compatible with it (e.g. benzoic acid) and to exploit its advantages. Nevertheless, it is evident that the generalized use of derivatization both for identification and quantitation of components in a complex mixture of vegetable origin still requires further developments. Few mass spectra and $I^2_S$ of TMS derivatives are available from the commercial libraries (NIST, etc.), thus making difficult component identification if reference standards are not available. These data can only be found in dedicated articles [18] or in studies concerning species belonging to the same genus or, as in this case, from products directly derived from the same plant, e.g. poplar based propolis [36,37].

REFERENCES
12. Isidorov VA, Vinogorova VT (2003) GC-MS analysis of compounds extracted from buds of *Populus balsamifera* and *Populus nigra*. Z Naturforsch 58:355-360
3.3 *Possible alternative technique to LC Chromatography*
Recently, there has been a rekindling of renewed interest in SFC especially thanks to renovate systems. Nowadays it is possible to obtain results comparable with the other latest developed more recent analytical techniques.

In this section, fifty compounds, all belonging to plant domain, are analyzed with comparable analytical conditions by UHPLC and UHPSFC techniques to highlight the advantages and disadvantages of both of them. A more precise study is done by SFC technique to test if it could replace LC or if it is able to give additional information to other analytical techniques on the same analyzed sample.

In UHPSFC analyses different parameters are tested to have the best conditions to analyze plant compounds:
- additives,
- columns.

Pyrethrum extract, was analyzed by UHPSFC to see the behavior of this technique with real samples and results compared with UHPLC technique.
ABSTRACT

Plant are considered to be an important resource to discover and develop new molecules with pharmaceutical activity. Thanks to the recent development in supercritical fluid chromatography (SFC), this technique has aroused again interest. The goal of the present study was to find general conditions to analyse plant extracts. Fifty plant standard compounds were chosen as sample. As the range of properties of plant compounds is very wide, four different additives were tested to find the best one. Many differences rose among the four both in power of elution, retention time and peak shape of the compounds. Column chemistry is another important parameter to test. Five different columns were tested with the best additives found. The addition of 5% of water to methanol and 2-ethylpiridine and HSS C18 SB columns seemed to be the best solutions. Finally, a real samples was analysed both with SFC and liquid chromatography (LC) to highlight both the advantages and disadvantages of SFC compared to the election techniques in the case of real samples.

KEYWORDS: UHPSFC, plant compounds, SFC additives, UHPLC.

1. INTRODUCTION

Since a long time, natural products provided the inspiration for a large number of molecules to treat many human diseases [1,2]. Even if during the past three decades the research of new pharmaceutical compounds among natural products has declined, they still remain a profitable field where to search for new molecules. This drop is due to different reasons: the easy and rapid development of different, but structurally related molecules against defined molecular targets for high-throughput screenings (HTS) thanks to the combinatory chemistry, the time-consuming and limits of conventional techniques to extract, isolate and elucidate new plant structures, the developments in molecular and cellular biology and genomic, which both increased the number of molecular targets and shortened the time of drug discovery and finally the decrease of interest of the major pharmaceutical industries for infection disease therapies [1-3]. Recently, a renewed interest in natural product discovery occurred, thanks both to the not completely satisfying HTS technique and procedure, instrument improvements and automation [3]. The advantage, but, at the same time, the disadvantage of natural products is their heterogeneity. Being already evolutionary selected and tested by nature, it is highly probable that they, and their related structures, have some biological role even in human beings. It is however difficult to predict their behaviour. Larsson and co-workers [3] tried to describe the natural products’ chemical space to discover more easily new possible active compounds. Chem GPS-NP was a tool able to chart biologically chemical space, provided to select high-probability hits and to predict their properties and activities. It considered more parameters, namely 35, for natural products than for pharmaceuticals to better describe their behaviour, such as molecular weight, lipophilicity, polarity, hydrogen bond capacity, flexibility, rigidity, etc.

Several chromatographic techniques, such as high-performance liquid chromatography (HPLC), gas chromatography (GC), capillary electrophoresis (CE), supercritical fluid chromatography (SFC) or thin layer chromatography (TLC), can be applied to solve natural mixtures. HPLC has been recognized to be one of the most useful and versatile techniques and it is used for this purpose until 1980 [4-7]. GC is a very advantageous technique, but limited for volatile and non-thermolabile compounds. SFC had a great fame and interest for a decade during the Nineties [4,6,8-11], but the other techniques grew more rapidly and shadowed it. Nowadays, thanks to the advent of redeveloped instruments, such as Waters Acquity UPC² system (which stands for Acquity UltraPerformance Convergence Chromatography™), that take advantages of the sub-2 µm particles, the interest in SFC technique has roused again.
There are many advantages of using SFC compared to LC and GC techniques, such as using carbon dioxide CO₂, that is an inexpensive and environmental friendly solvent, together with the lower use of toxic organic solvents, as only small amount of organic modifiers are added to CO₂ to improve peak shape, the combined high viscosity and diffusivity of supercritical fluids, the easy control of solvent strength by simply tuning pressure and/or temperature and the possibility to switch from chromatographic to extraction methods, with all the advantages of the SFE (supercritical fluid extraction) technique.

Preparative HPLC was the preferred technique in the past 20 years [12], since a generic gradient was able to reduce method development time and purification, but different works showed the benefits of SFE, especially in plant extraction [6,8-11,13]. The lower viscosity of the mobile phase leads to work at higher flow rates compared to HPLC, resulting in shorter analysis time and product recovery is usually higher than conventional method extractions, such as Soxhlet, steam distillation, percolation and maceration techniques [4,12,13]. Moreover, SFE is suitable too for food applications thanks to the CO₂ non-toxic character and the small amount of solvent to remove [14].

CO₂ presents however some limitations: because of its low polarity, it is not able to extract from matrix and to elute from the column polar compounds, so small percentage of polar modifiers must be added to increase extraction and elution power [6,10].

The first aim of this work is to find the best and more general analytical conditions for a pool of different plant compounds in SFC testing both different additives in the organic modifier and different column chemistries. The second aim is to compare results of the fifty plant compound pool obtained in SFC with the ones obtained with other analytical techniques normally employed for natural compound separations, such as LC. Finally, real plant samples, such as standardized Pyrethrum extract, are analysed with rapid SFC and LC techniques to highlight both the advantages and limits for each approach.

2 EXPERIMENTAL
2.1 Chemical and reagents
LC-MS grade solvents (methanol and isopropanol) were purchased from VWR (Radnor, PA, USA). Heptane (99%, extra dry) was purchased by Acros (Geel, Belgium). Acetonitrile, water, acetic acid glacial and formic acid (ULC-MS grade) were purchased from Biosolve BV (Valkenswaard, The Netherlands). Ammonium hydroxide (28%, w/v NH₃ in water ≥ 99.99% metals basis) was purchased from Sigma-Aldrich (Buchs, Switzerland). CO₂ was provided by Pangas (Dagmersellen, Suisse).

A pool of 50 different plant analytes was chosen: 2-hydroxycinnamic acid, 3,4-dimethoxycinnamic acid, Ascorbic acid, Benzoic acid, Caffeic acid, Carnosic acid, Catechin, Cinnamic acid, Coumaric acid, Epicatchin gallate, Epigallocatechin, Epigalloctechin gallate, Gallocatechin, Gallocatechin gallate, Isoferulic acid, Naringin, Pyrethrum extract, Quercetin, Syringic acid and Tyrosol were obtained from Sigma-Aldrich (Milan, Italy). Chlorogenic acid Fumaric acid Rosmarinic acid were purchased from Sigma-Fluka (Buchs, Switzerland). Eriodictyol and Protocatechuic acid were provided by Extrasynthése (Genay Cedex, France). Cyanidin and Cyanidin-3-O-glucoside were gently offered by Indena (Milan, Italy). Anthrone, Arbitin. Brucine Cinnamaldehyde Colchicine, Curcumin Digitoxin, Diosgenin, Emodian, Epicatechin Ferulic acid, Gallic acid Glycyrrhetinic acid, Hederagenin Isorhamnetin, Kaempferol, Khellin, Naringenin Plumbagin Reserpine, Rutin, Sinapic acid Theobromine Vanillic acid were gently offered by prof. Wolfender.

Stock standard solution of each analyte was prepared by dissolving each reference compound in isopropanol to obtain a concentration of 1 mg/mL (1000 ppm), except for Theobromine, Isorhamnetin, Catechin, Epicatechin, Epicatchin gallate, Epigallocatechin, Epigalloctechin gallate, Gallocatechin, Gallocatechin gallate, Reserpine and Rutin that were dissolved in methanol and Glycyrrhetinic acid in ethanol. Standard solutions at desired concentrations were
Analyses of plant extracts by Supercritical Fluid Chromatography: first approaches

prepared by diluting stock solutions. In UHPSFC, dilutions of compounds were prepared at 50 ppm in isopropanol, while in UHPLC they were prepared at 25 ppm in pure water. Pyrethrum standardized extract Pestanal® was purchased from Sigma–Aldrich (Milan, Italy) and stock solution was prepared in pure methanol.

2.2 Instrumentation

2.2.1 UHPSFC system
The Waters Acquity UPC² system (which stands for Acquity UltraPerformance Convergence Chromatography™) was equipped with a binary solvent delivery pump that possessed a 250 µL mixing chamber and was compatible with mobile phase flow rates up to 4 mL/min and pressures up to 414 bar (upper pressure limit was 414 bar at 3.25 mL/min and 293 bar at 4 mL/min), an auto-sampler with a fixed-loop volume of 10 µL loop, a column oven compatible with temperatures up to 90°C, and a UV detector that included an 8 µL flow-cell and a backpressure regulator (BPR). UV spectra were recorded from 210 to 400 nm with a sampling rate of 20 Hz and a filter time constant of 0.05 sec. The connection tube between the injector and column inlet was 600 mm long (active preheater included) and had an I.D. of 0.175 mm, and the capillary located between the column and detector was 600 mm long and had an I.D. of 0.175 mm. The extra-column volume of this instrument was estimated to be 59 µL, and the system dwell volume was 440 µL.

2.2.2 UHPLC system
Experiments in UHPLC-DAD were performed on the Waters Acquity UPLC I-Class System (Milford, MA, USA). This instrument included a binary solvent delivery pump that possessed a 50 µL mixing chamber and was compatible with a maximum flow rate of 2 mL/min and backpressure up to 1240 bar (upper pressure limit was 1240 bar at 1 mL/min and 827 bar at 2 mL/min), an auto-sampler with a fixed-loop volume of 10 µL in partial loop with needle overfill conditions, a column oven compatible up to 90°C and a UV detector that include a 0.5 µL flow-cell. UV spectra were recorded from 210 to 400 nm with a sampling rate of 20 Hz and a filter time constant of 0.05 sec. The oven temperature was maintained at 40°C. The connection tube between the injector and column inlet was 250 mm long (passive preheating included) and had an I.D. of 0.13 mm, and the capillary located between the column and detector was 150 mm long and had an I.D. of 0.1 mm. The extra-column volume of this instrument was estimated at 14 µL and the system dwell volume was equal to 90 µL.

2.3 Software
Data acquisition and control of the UHPLC systems were performed using the Waters Empower™ Pro 2 Software. The UHPSFC system was controlled with the Empower™ Pro 3 Software.

2.4 Columns
UHPSFC separations were carried out on various analytical columns: Acquity UPC²™ BEH 2 EP (100 x 3.0 mm ID, 1.7 Å), Acquity UPC²™ HSS C18 SB (100 x 3.0 mm ID, 1.7 Å), Acquity HSS Cyano (75 x 3.0 mm ID, 1.8 Å), Acquity CSH Fluoro-Phenyl (100 x 3.0 mm ID, 1.7 Å) and Acquity UPC²™ BEH (75 and 100 x 3.0 mm ID, 1.7 Å).

UHPLC separations were carried out on various analytical columns: UPLC HSS C18 (50 x 2.1 mm ID, 1.8 Å), Acquity UPLC BEH C18 (50 X 2.1 mm ID, 1.7 µm), Acquity UPLC BEH C18 Shield (50 X 2.1 mm ID, 1.7 µm), Acquity UPLC BEH C18 (50 X 2.1 mm ID, 1.7 µm), Acquity UPLC BEH Phenyl (50 X 2.1 mm ID, 1.7 µm) and Halo C18 (50 X 2.1 mm, 2.7 µm).
3 RESULTS AND DISCUSSION

During last years, general attitudes are to make greener and greener chemistry. Lately, SFC gained interest again thanks to its low impact on the environment. Nowadays only few applications are reported in literature in analytical domain. This technique is especially used for pharmaceutical and enantiomeric separations and for preparative extractions. In plant domain, SFC is barely exploited because of the unsuitable characteristics of the molecules and the complexity of extracts.

3.1. Evaluation of various additives

Moderate critical conditions of pressure and temperature, together with the low toxicity and price, make CO₂ the solvent of election for SFC analysis. However, it presents the disadvantage to have non-polar properties, that means that SFC can be considered as a normal phase technique, so unsuitable for polar compounds. This disadvantage can be overcome by adding organic modifiers to CO₂ from very low (2-5%) up to very high concentrations (50-60%) [15]. It is possible to choose among a large number of solvents, such as methanol, isopropanol, butanol, ethanol, dimethylsulfoxide, acetonitrile, etc. [16]. Usually, acetonitrile is barely used, as it is the less eco-friendly solvent, and so it reduces the green attitude of the technique [17]. When small concentrations of such polar solvents are added to CO₂, solvent strength of the mixture increases dramatically. This is caused not only by the increase of density of the mobile phase, but also by changes of its polarity and interactions with the stationary phase of columns [16,18].

Even with addition of organic modifiers, it is still possible that the most polar or basic compounds did not elute correctly from the column. Therefore, it is necessary to add a third component to the mobile phase, the additive. It is a compound with acidic or basic properties chosen according to the nature of the analytes. Generally, acidic additives are used for acidic solutes, while basic additives for basic solutes. Conversely to the modifier, the additive is added in very small amounts, <1% v/v, and it allows the increase of efficiency and the improvement in peak shape [16]. Many articles report that additives can act in different ways: i) cover the active sites of the stationary phase, ii) change the polarity of the stationary phase, iii) suppress the ionization of the analytes, iv) enhance ion pair formation with the analytes and v) increase the polarity and solvating power of the mobile phase [19,20]. In literature, several additives are used to enhance separation, such as trifluoroacetic acid, citric acid for acidic compounds and isopropylamine, diethylamine, triethylamine, ethyldimethylamine for basic compounds [16,21], but not all of them are mass (MS) compatible or easy to remove after separation. More recent studies reported the use of ammonium hydroxide as a potential substitute for basic additives, since it is MS compatible and volatile under ambient conditions [16,22,23].

In the first section of this study, the goal was to find the best additive to add to MeOH/CO₂ mixture. As UHPSFC could be coupled with MS spectrometry, all the chosen additives must be MS compatible for further studies. Plant compounds have large spectrum of propriety in term of polarity, molecular weight, lipophilicity, hydrogen bond capacity, etc. For this reason, additives with different properties such as formic acid (FA), ammonium hydroxide (NH₄OH) and water, were added to the modifier, MeOH, to investigate which one among the solvent mixtures gave the best peak performance. Water is an anomalous additive in SFC technique, but its addition can improve peak shape of polar compounds and extend the polarity rate of the columns, thanks to the higher H-bonding capacity of water compared to MeOH [15,19]. Water has a very low solubility in CO₂ (~0.1% w/w), so it is necessary to add small amounts directly to MeOH and, because of the formation and dissociation of carbonic acid, it makes acidic mobile phase pH. All the fifty compounds were analysed with the Acquity UPC²™ BEH column with five different mobile phases, namely pure MeOH, MeOH + 20 mM FA, MeOH + 20 mM NH₄OH, MeOH + 20 mM FA + 20 mM NH₄OH and MeOH + 5% water. The choice of using basic and acidic additives together derives from precedent studies carried out in the laboratory.

Chromatograms of six peaks, chosen among the pool of the fifties to highlight the advantages and limits of each additive, are reported in fig. 1. It is evident how the additive can modify peak
shape, retention and even allow elution for some analytes, while it is totally non-influential for others. In all of the five conditions, there were no problem of elution for colchicine: the retention time was hold and the peak appeared always thick and symmetrical. Theobromine did not show time shifts in the five chromatograms, but peak shape improved by using NH₄OH, water and partially with both acidic and basic additives. In both chromatograms where FA was used, the baseline increased, but this effect was due to the presence of the acid that adsorbs in the region close to the maximum of the spectrum of theobromine. No condition of works was able to elute naringenin with a good peak shape. The addiction of basic additive and, in particular, with the presence of the acidic additive too, the peak was thicker, even if it continued to present an important asymmetry.
Analyses of plant extracts by Supercritical Fluid Chromatography: first approaches

Retention time of cinnamic acid was subjected to evident time shifts, especially in basic condition. Peak shape was comparable using pure MeOH, water and FA, while it improved with basic additive and it was not acceptable at all for the acidic/basic mobile phase. The rutin is the compound that presents the highest number of H-bonding among the group of chosen compounds. In fact, this analyte did not elute with any mobile phase condition. This is a very interesting remark, as elution in SFC is not only limited by the polarity, but it is also strong influenced by H-bonding power of the molecules. Water was the only additive that allowed the elution of epicatechin (fig. 1 E, peak 3), even if the peak did not have a correct shape.

As reported in fig. 2 E, water is the additive that gave the best performances in term of number of eluted peaks with good and bad peak shape.
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Figure 2: Peak shape obtained with the five organic mobile phases: A) MeOH, B) MeOH + 20 mM Formic Acid, C) MeOH + 20 mM Ammonia, D) MeOH + 20 mM Formic Acid + 20 mM Ammonia, E) MeOH + 5% H₂O. Analyses were performed on Acquity UPC²™ Beh (75 x 3.0 mm ID, 1.7 µm) column.

Pure methanol and acidic condition gave the same results: half compounds did not elute from the column and only one-fifth had an appropriate shape. The addition of NH₄OH gave results only slightly lower than water for both number of eluted peaks and peaks with good shape. Finally, the addition of both acidic and basic additive allowed to elute the same number of compounds as pure MeOH and acidified MeOH, but the peak shape was improved by 40%.

3.2. Evaluation of various column chemistries

In the case of SFC, the choice in terms of mobile phase is rather limited because of solubility/miscibility issues, possible precipitation of salts under supercritical CO₂ and impossibility to control precisely mobile phase pH, like in RPLC. For these reasons, the stationary phase plays a major role for tuning selectivity and adjusting retention of compounds, while the mobile phase is generally optimized to attain suitable peak shape on lead elution, as demonstrated in §3.1. As shown in figures 1 and 2, the results achieved with the hybrid bare silica were relatively poor, even in presence of various types of mobile phase additives.

Since the present study was carried out exclusively using columns packed with sub-2 µm particles under SFC conditions (UHPSFC), the number of existing column chemistries is restricted compared to conventional SFC. Thus, only five commercially available stationary phases were tested using only binary mixtures of MeOH/CO₂ as mobile phase or MeOH/CO₂ combined with the most promising additives, namely 5% water and 20mM NH₄OH. The five selected columns, namely bare hybrid silica (BEH), hybrid silica bonded with 2-EP moiety (BEH 2-EP), silica bonded with cyano moiety (HSS CN), silica bonded with C18 alkyl chain (HSS C18 SB) and charged surface hybrid silica bonded with fluoro-phenyl (CSH FP) were quite different in terms of dipolarity, polarizability, H-bond donor (acidity), H-bond acceptor (basicity) and molar volume [24]. It is thus expected that retention, selectivity and probably peak shape using these different phases would be quite different. This has been recently demonstrated using drugs
as model compounds [25], but it has never been reported with natural substances, having challenging physicochemical properties and a wide polarity range, as illustrated in Table 1.

To limit the number of experiments that need to be conducted, a restricted set of compounds containing only 10 natural substances was selected to screen the five different stationary phases and the three mobile phase conditions. The selection of the 10 representative compounds was based on the experiments performed in §3.1., with the different additives. Among the 10 compounds, three of them presented a suitable peak shape and retention time, three of them were distorted (severe broadening, tailing or fronting was observed) and the last four compounds were not eluted, using the bare hybrid silica phase.

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)
Analyses of plant extracts by Supercritical Fluid Chromatography: first approaches

Figure 3: Chromatograms of six representative compounds: 1) Epicatechine, 2) Colchicine, 3) Naringenin, 4) Emodin, 5) Syringic Acid, 6) Theobromine. Analyses were performed on A) Acquity UPC™ BEH 2 EP (100 x 3.0 mm ID, 1.7 µm), B) Acquity UPC™ HSS C18 (100 x 3.0 mm ID, 1.7 µm), C) Acquity HSS Cyano (75 x 3.0 mm ID, 1.8 µm), D) Acquity CSH Fluoro-Phenyl (100 x 3.0 mm ID, 1.7 µm), E) Acquity UPC™ Beh (75 x 3.0 mm ID, 1.7 µm) columns. Solvent gradient was kept for 0.75 minutes at 2% and then increased to 40% of the organic mobile phase MeOH + 5% H2O in 7.5 minutes for 75 mm column length and for 1 minute at 2% and then increased to 40% of the organic mobile phase MeOH + 5% H2O in 9 minutes for 100 mm column length, both with a flow rate of 1.5 mL min⁻¹. Column temperature was 40°C and injected volume 1 µL. ABPR pressure was kept at 150 bar. UV spectra were recorded from 210 to 400 nm with a sampling rate of 20 Hz and a filter time constant of 0.05 sec.

Then, the 10 compounds were individually injected using the 15 different conditions - 5 stationary phases and 3 mobile phase conditions - and some representative chromatograms of 6 compounds were reported in fig. 3 with mobile phase containing 5% water, while the average asymmetries and peak widths were given in fig. 4.
As expected, some significant differences were observed in terms of selectivity, peak shape and retention when changing the chemical nature of the stationary phase. Fig. 3 shows that the performance achieved with the BEH 2-EP, HSS C18 SB and CSH FP were acceptable, compared to that obtained with the BEH and HSS CN, but the epicatechin could not eluted from the CSH FP column. Fig. 4 confirms this ranking, using figures of merit (average asymmetries and peak widths for the 10 model compounds). In terms of peak widths (fig. 4B), the thinnest peaks were generally attained with the BEH 2-EP, HSS C18 SB and CSH FP phases, except when combining the HSS C18 SB stationary phase with a mobile phase containing NH₄OH (in these conditions, syringic acid was particularly broaden and increases the average peak width value). In contrast, the peaks observed on the HSS CN and BEH were significantly broader whatever the mobile phase additive. Regarding asymmetry (fig. 4A), the columns were almost equivalent, with average values for the 10 compounds comprised between 1 and 2. Only the CSH FP phase provides a notably higher tailing (average asymmetry at 10% comprised between 2 and 3). The strong tailing observed on the CSH FP material can be related to the presence of positive charges at the surface of the silica (charged surface hybrid phase) and the nature of the investigated natural substances (mostly weak acids, possessing several phenol and/or carboxylic acid moieties). Based on these observations, the HSS C18 SB and BEH 2-EP were the most appropriate ones in terms of asymmetry and peak width. Except peak shape, it is also important to consider the retention of the investigated compound. Indeed, it was previously demonstrated (§3.1.) that 44 to 50% of the natural substances could not be eluted from the BEH phase, whatever the mobile phase additive, because of strong H-bonding interactions. Thus, the possibility to elute some compounds possessing a high number of H-bond donor and H-bond acceptor groups should be considered. Indeed, retention in UHPSFC is mainly driven by H-bond interaction and by π-π interactions as well as polarity, to a lesser extent.

As shown in fig. 3C, the HSS CN phase provides distorted peaks for all the natural substances reported here and is probably the worst column for this class of compounds that have challenging physicochemical properties and a wide polarity range. According to the provider, this phase is...
Analyses of plant extracts by Supercritical Fluid Chromatography: first approaches

non-endcapped and contains a monofunctional cyanopropyl ligand bonded to silica. Because of its intrinsic properties and since the bonded group has a limited steric hindrance, the amount of accessible silanols is probably high on this material. Thus, there are various types of interactions that should coexist, including dipole-dipole and hydrogen bond, and could explain peak broadening. In addition, the syringic acid (peak 5) could not be eluted from this phase, probably because it possesses a strong dipolar moment together with numerous H-bond donor/acceptor groups.

As reported previously, the BEH phase, corresponding to the bare hybrid silica, also provides poor performance for the analysis of natural substances (see fig. 3E). The retention was too high for polar compounds possessing high H-bond capability (e.g. epicatechin not eluted) and peaks were strongly distorted.

On the other hand, the BEH 2-EP material allows the elution of the 6 compounds reported in fig. 3A, with suitable peak shape. This behaviour can be explained by the nature of the bonding and its lower possibility of H-bond compared to BEH and HSS CN phases. Indeed H-bond interactions are reduced because of possible intermolecular hydrogen bonding between the nitrogen atom in the pyridine group and the residual silanol groups of the silica, since the BEH 2-EP is not endcapped. However, because of the strong affinity between the basic stationary phase and the acidic analytes, some acids (most of the natural substances present some acidic properties) cannot be analysed successfully using the BEH 2-EP column [26].

The HSS C18 SB also appears as a suitable phase for analyzing natural products (see fig. 3B). Indeed, this phase is not endcapped and possesses an intermediate coverage of silica matrix with trifunctionally bonded C18 moiety. In other words, it presents a significant number of residual silanols which are partially masked by the alkyl chains. For this reason, the H-bond capability of the material is probably reduced compared to the HSS CN and BEH and the elution order should be different from that obtained with the other stationary phases. As example, the epicatechin was the most retained compound on the other phases but was one of the less retained compounds on the HSS C18 SB. In addition, the peak shape appears to be suitable, even if the peaks were slightly broader compared to those obtained on the BEH 2-EP phase. To further reduce the amount of residual silanols and elute the most retained natural substances, some additional experiments were conducted with a fully endcapped hybrid C18 material (Acquity BEH C18).

Last but not least, the chromatographic profiles obtained with the CSH FP (fig. 3D) was in between those of HSS CN and BEH material (unacceptable performance) and that of BEH 2-EP and HSS C18 SB (best performance). With this CSH FP phase, the peaks were very thin but tailing was often more pronounced than on the BEH 2-EP or HSS C18 SB. In addition, because of the positive charge at the surface of the hybrid silica matrix, the compounds presenting high acidity were strongly retained. Except this positive charge, some π-π interactions as well as electron donor/acceptor complex (i.e. electrostatic interaction in which the charge is transferred from an electron donor to electron acceptor) can also be created between the fluorophenyl group and the phenol moiety contained in many of the natural substances. These statements explained why epicatechin was not eluted from the CSH FP material. Indeed, the latter is an acidic compound (pKa around 9.5), quite polar (logP of 0.61) and is able to create numerous π-π interactions and electron donor/acceptor complex because of the presence of two catechol moieties.

3.3. Evaluation of the best analysis conditions (UHPSFC and UHPLC)

As demonstrated in §3.1. and §3.2., among the columns packed with sub-2µm particles and dedicated to UHPSFC experiments, the BEH 2-EP and HSS C18 SB columns appeared to be the most promising material. Moreover, the addition of a small percentage of water to MeOH was found to be optimal to decrease peak broadening, asymmetry and elute the most polar and strong H-bond donor/acceptor compounds from the column. All these conditions have been applied only on a small selection between the pool of compounds, therefore to confirm the trend of the
analyses, all the fifty compounds were analysed with the optimal conditions: BEH 2-EP and HSS C18 SB columns and 5% of water added to MeOH as mobile phase. The pie charts reported in fig. 5 show immediately how important is the choice of the right column chemistry in SFC, as described in §3.2.

**Figure 5:** Peak shape obtained with three columns: A) Acquity UPC2™ Beh (75 x 3.0 mm ID, 1.7 µm), B) Acquity UPC2™ Beh 2 EP (100 x 3.0 mm ID, 1.7 µm) and C) Acquity UPC2™ HSS C18 (100 x 3.0 mm ID, 1.7 µm). Solvent gradient was for 1 minute at 2% and then increased to 40% of the organic mobile phase MeOH + 5% H₂O in 9 minutes with a flow rate of 1.5 mL min⁻¹. Column temperature was 40°C and injected volume 1 µL. ABPR pressure was kept at 150 bar. UV spectra were recorded from 210 to 400 nm with a sampling rate of 20 Hz and a filter time constant of 0.05 sec.

It is possible to eliminate at a glance the BEH column, as the number of the eluted peaks decreased of the 20% ca. compared with the other two columns and only the 30% of the peaks had the correct shape. The selection between the BEH 2-EP and HSS C18 SB columns could be harder, as the percentage of eluted peaks is apparently the same. In reality, more than 40% of eluted peaks on the BEH 2-EP column did not present peak symmetry included in the chosen limits. Lastly, on the HSS C18 SB column, half of the chosen compounds eluted correctly and the 14% with asymmetry beyond the selected limits. But, even using this column, an important percentage of the fifty compounds, namely 36%, did not elute from the column. This is a limit, as in real samples it could be possible that not all the compounds present in the mixture eluted. As HPLC is considered one of the most useful and versatile analytical techniques in plant domain, all the fifty compounds were also analysed by liquid chromatography (LC) and compared to SFC results to highlight the limits and advantages of both techniques. To be totally comparable, the analyses were performed on a Waters Acquity UPLC 1-Class system with an Acquity UPLC HSS C18 column with the same particle dimensions, but reduced diameter, namely 2.1 mm, compared with the one used in SFC analyses. As the mixture CO₂/MeOH has pH of 5 ca., an appropriate pH 5 acetate buffer with pure MeOH was used in LC as mobile
phase. The column temperature was fixed at 40°C and UV parameters were unchanged compared to SFC analyses.
In LC, all the fifty compounds eluted correctly with a correct peak shape (data not shown).

As the two techniques exploit the opposite separation rules, retention factor (k) of each eluted compound in the two techniques was compared to find any possible correlation. In fig. 6, k of compounds analysed by UHPLC are plotted in function of k of the same compound analysed by UHP SFC. It is evident from the graph, that there is no correlation between the retention factor of the same compounds analysed by the two techniques, in fact the correlation points are spread all over the area. This is an interesting result, as the two techniques can be considered orthogonal. It is therefore possible to obtain a higher number of information on the same sample or confirm the obtained results by analysing them by LC and SFC.

3.4. Technique comparison on real samples
The preliminary study on the fifty standard plant compounds showed that not all of them can be correctly analyzed by SFC. The biggest limits are for those molecules that are very polar and/or present a high number of H–donor/acceptor bonds. To overcome these problems, it is possible to use end-capped columns to limit free silanols and so decrease interactions between the stationary phase and the molecules. If this possibility did not give satisfactory results, stationary phases with different chemistries or the addiction of small percentage of water or other additives, such as NH₄OH or FA, to enhance the polarity range of the column can give remarkable results.
In plant domain, until now supercritical fluids have been especially exploited for extraction more than qualitative and quantitative purposes. To evaluate the possibility to exploit this technique in analytical analyses too, two real samples were analyzed and the obtained results compared with LC. The chosen extract is: standardized pyrethrum extract. The pyrethrum extract is formed by natural organic esters derived from Chrysanthemum cinerariifolium. Six different compounds have been isolated and identified, namely Pyrethrin I and II, Cinerin I and II and Jasminol I and II. They have a potent insecticidal activity, as they attack the nervous systems of all insects, on the contrary they are totally harmless for human beings and mammals. They are non-persistent, being biodegradable, and break down on exposure to light, oxygen and high temperature. This
extract has a very non-polar character and all of the six compounds have a low number of H-donor/acceptor bonds.

For the two techniques, different mobile phases and column chemistries were tested to obtain the best possible performances in term of separation and time analysis. As the mixture is very simple, columns of 5 cm length were sufficient to attain the desirable separation. No additives were added to MeOH to increase the polarity range of the column for SFC analyses, since this sample presents very low polarity. Five different column chemistries, namely BÉH, BEH 2-EP, HSS CN, HSS C18 and SBCSH FP, were tested with the same instrumental conditions. Cyano and fluoro-phenyl columns were totally inappropriate to achieve a good separation, in fact many peaks co-eluted (data not shown). HSS C18 lead a complete separation of Jasmolin I from II, but only a partial separation of Pyrethrin and Jasmolin I and II and, moreover, 4 minute analysis ca. is not competitive for only six peaks. The resolution of the two critical pairs was enhanced on the BEH column, but it remained critical and not completely satisfactory, while analysis time decreased of 25%. Finally, 2-EP was able to give the best separation in term of analysis time and resolution: all the six peaks were well resolved in only 2.50 min, as fig. 7 A reported.

Figure 7: comparison of the analyses in UHPSFC and UHPLC of pyrethrum extract. A) Acquity UPC™ Beh 2 EP (50 x 3.0 mm ID, 1.7 µm) column, B) Acquity Beh Phenyl (50 x 2.1mm ID, 1.7µm) column.

To be comparable with the work done on UHPSFC system, LC analyses were conducted on the same UHPLC system used for the former analyses. First, different organic mobile phases were tested: methanol and acetonitrile pure and added with different organic modifiers, such as FA and buffer of ammonium acetate. It was better to work with acetonitrile, as it generated lower backpressure, being less viscous than methanol, and it was not necessary to add modifiers to the organic mobile phase to obtain a correct peak shape, as none among the six analytes presented ionisable functions (data not shown). Second, three steps of temperature were tested, namely 30, 45 and 60°C. As described in literature [27], backpressure and analysis time decreased progressively increasing the temperature. The intermediate temperature, 45°C, was chosen to continue the study, as it was able to reduce backpressure of 20% and analysis time of 15%, but it did not stress the column as higher temperature. Third, two different column technologies were tested: core-shell and sub-2 µm. Both columns gave good separation, but sub-2 µm column generated a higher backpressure compared with the core-shell one. In this case, it is not a
problem, as the UHPLC system can withstand backpressure until 1000 bar. Sub-2 µm column was therefore chosen, as there are many more chemistries available on the market, compared to core-shell technology. Four different chemistries were tested: BEH C18, BEH C8, BEH C18 Shield and BEH Phenyl. BEH C18 and BEH C8 presented both good separation, as the two stationary phases presents very close chemistries, but the analysis lasted 5.70 and 3.8 min respectively. BEH C18 Shield presents a carbamate group that gives to the column a higher resolution power, especially for those compounds that present close chemical features, compared to BEH C18 and BEH C8 columns. Analysis time is lowered of about 45%, keeping the same good separation (data not shown). The best performance was reached by BEH Phenyl column. This column exploits $\pi-\pi$ interactions between the aromatic cycles presents on the stationary phase with the aromatic cycles or conjugated systems present on the analysed molecules. Analysis time was further on decreased of 20% compared to BEH C18 Shield analysis, while separation improved (fig. 7 B).

4 CONCLUSIONS

In the present study, plant samples were analysed by UHPSF to evaluate advantages and disadvantages of this technique compared to LC, that is the technique of choice for this type of analytes. A pool of fifty compounds were chosen as representative of plant compounds. First of all, several additives, such as water, ammonia and formic acid, were added to methanol and all the fifty compounds tested with a bare silica column. It is evident how important the influence of the additive is both on the elution of the compounds and peak shape. Water and ammonia resulted to be the best additives. It is surprising the effect of water, but it is able to increase the polarity of the mobile phase and to create new H-H bonding between the mobile phase and analysed molecules.

Second, several stationary phase columns were tested, namely BEH, BEH 2-EP, HSS CN, HSS C18 and SBCSH FP, on a selected number of compounds with the two best additives. BEH, HSS CN and SBCSH FP gave the worst results, so to obtain a more general view, all the fifty compounds were analysed on the two best columns, BEH 2-EP and HSS C18 with the final best additive, water. Both columns were not able to elute the totality of the compounds, but HSS C18 resulted to be the best for the peak shape. All the fifty compounds were analysed by UHPLC, to compare the retention factors of both techniques. There is absolutely no correlation between the two techniques, so it is possible to use them to increase the number of information or to confirm results.

Finally, a real sample was analysed, the pyrethrum extract. Since this extract is very non-polar, no additives were added to the mobile phase. The BEH 2-EP column resulted to be the best column in term of efficiency and analysis time. Optimized LC analysis was compared with SFC analysis: there is no evident advantages of using SFC except for the lower use of organic solvent. In conclusion, for the pool of fifty standard compounds, water resulted to be the best additive and SSS C18 the best stationary phase column, but it is not possible to generalize, as the analysis of the real sample showed.
5 REFERENCES

4. Conclusions
Plant extracts are often very complex to be analyzed because of both the high number of compounds and their structural differences. Several analytical techniques are used, but LC appears to be the best one for non-volatile compounds.

Different approaches are available in plant analyses, such as metabolic fingerprint, metabolic profiling, dereplication and targeted analysis and for each of them, analytical methods with different characteristics are required.

Fast analyses give the required information for metabolic fingerprint and profiling, while high efficiency with very long gradients are required for dereplication and targeted analyses.

Many new techniques could be usefully employed to reach this goal, including sub-2 µm and core-shell particle columns, high temperature analyses and sensitive detectors.

The first step is the optimization of different parameters to obtain the best separation. The column chemistry and pH can give impressive results by changing selectivity and therefore by improving separation. High temperature is very interesting, as it is useful to decrease the backpressure, but it does not drastically influence selectivity.

Core-shell column efficiency is comparable to sub-2 µm columns, but the generated backpressure is lower and acceptable for conventional HPLC instrumentation. A sensitive detector, such as QqTOF/MS, not only effectively contributes to unknown compound identification, but also to overcome possible limitations in chromatographic separations, since their sensitivity is higher and its response is more diagnostic compared to an UV detector. The latter can be adopted instead when the investigated extract is very complex and very sensitive detectors are not available. Efficiency can also be increased by coupling more than one column and by applying high temperature to decrease the resulting backpressure to enable the adoption of the optimal flow rate. These new approaches requires long analysis times, but efficiency is dramatically increased.

Another analytical technique, UHPSFC has been tested to highlight possible advantages or disadvantages compared to UHPLC. Different additives and columns have been tested to find the optimal working conditions. Water was the most effective additive and 2-EP the best column for the pool of investigated plant compounds. UHPSFC results to be less effective than HPLC for their separation. Pyrethrum extract was taken as a model real-world sample, and required different UHPSFC conditions compared to those adopted for UHPLC. Further studies and columns are necessary on this subject.

New column technologies were proved to be successful to improve separation of very complex matrices. It is however advisable to continue to test core-shell columns from different brands and with different chemistries to find the most appropriate one. The combination of advanced chromatography with modern MS detectors has still to be investigated in order to obtain the information that they can provide. Special attention should be paid to quantitative analyses with UHPLC-MS/MS in view of the ever stricter rules introduced by the European Community on natural products. On the other hand, UHPSFC requires a strong effort to develop new dedicated stationary phases in order to make this technique complementary but orthogonal to modern LC.