Published in : Journal of Pharmaceutical and Biomedical Analysis (2022), 221, p. 115071
DOI: 10.1016/j.jpba.2022.115071
Status : Postprint (Author's version)

EMERGING ANALYTICAL TECHNIQUES FOR PHARMACEUTICAL QUALITY CONTROL: WHERE ARE WE IN 2022?

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Keywords: Multidimensional chromatography; NIR spectroscopy; Pharmaceuticals; Quality control; Raman spectroscopy; Supercritical fluid chromatography; Vibrational spectroscopy

Abstract

Quality control is a fundamental and critical activity in the pharmaceutical industry that guarantees the quality of medicines. QC analyses are currently performed using several well-known techniques, mainly liquid and gas chromatography. However, current trends are focused on the development of new techniques to reduce analysis time and cost, to improve the performances and decrease ecological footprint. In this context, analytical scientists developed and studied emerging technologies based on spectroscopy and chromatography. The present review aims to give an overview of the recent development of vibrational spectroscopy, supercritical fluid chromatography and multi-dimensional chromatography. Selected emerging techniques are discussed using SWOT analysis and published pharmaceutical QC applications are discussed.

1. Introduction

Medicines quality, safety and availability is a major concern to guarantee public health. Regulatory agencies ensure drug quality, efficacy and safety thanks to guidelines and requirements, especially the Good Manufacturing Practices (GMP) guidelines [1]. Among GMP requirements, quality control (QC) is a keystone of drug quality. It encompasses all steps of pharmaceutical manufacturing, from the control of raw materials (i.e., drug substances and excipients) to the release of the drug product (i.e., the medicines that will be administrated to the patient). Next to the pre-marketing phase, the post-marketing QC by national and international regulatory agencies (e.g., WHO) is also an important guarantor of drug quality.

One of the main objectives of QC is to identify and quantify the active substance(s) and to track impurities by means of several analytical techniques, including separation and spectroscopic techniques. Based on European and US Pharmacopeias, liquid chromatography is the predominant analytical tool used for QC analysis. It could also be extrapolated to the overall pharmaceutical environment where GC, HPLC and UHPLC, with or without MS detection, remain the most often selected techniques for a wide range of pharmaceutical applications [2].

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Throughout history, scientists tried to improve analytical technologies, methods, and instrumentations. The innovation process always follows the main objective to provide faster, greener, less expensive, more efficient and/or more sensitive analytical tools. Next to these technological innovations, it is important to keep in mind that the implementation of new technologies or the modification of current methods for pharmaceutical QC remains challenging. Indeed, several regulatory aspects should be carefully fulfilled and well described to accept new techniques or methods. In this context, the aim of this review is to present some emerging analytical technologies and to evaluate their potential for pharmaceutical QC, in respect with the current regulatory requirements. The discussion is focused on analytical techniques dedicated to small molecules analysis, mostly affected by using old- fashioned analytical methods. Both spectroscopic and separation techniques are described using SWOT analysis (strengths, weaknesses, opportunities, and threats). Near-infrared and Raman spectroscopies are discussed in the part related to spectroscopic techniques. Regarding the separation techniques, supercritical fluid chromatography (SFC) and multi-dimensional chromatography are considered. Discussion on their applicability for the QC of small molecules is also proposed. The present paper discusses some relevant aspects and exemplary applications of the last five years (2017–2022), without making an exhaustive review of the literature.

2. Vibrational spectroscopic techniques

The most used vibrational spectroscopy techniques for the pharmaceutical QC are the near-infrared (NIR) and Raman spectroscopies. These techniques rely on different but complementary principles: NIR spectroscopy is based on the absorption of light by asymmetric polar bonds of the sample. The Raman spectroscopy arises from the inelastic scattering of light by the symmetric nonpolar bonds of the sample molecules.

2.1. SWOT ANALYSIS OF NIR [3–8]

The strengths of NIR originate from the advantages of vibrational spectroscopy. NIR is green and non-destructive having a fast data acquisition. Samples can be very quickly analyzed without any sample preparation implying that NIR is a useful tool for real-time analysis (e.g., online and inline control of manufacturing). Moreover, several acquisition modes are available: transmission, reflection and transflection. Instrument easiness of use is also another major strength of this technique.

The weaknesses of NIR are mainly related to the low sensitivity of the technique (around 1 % w/w). It means that NIR could not be used for low-dosed API or impurities determination. NIR encompasses the spectral region from 12,500 to 4,000 cm^{-1} (2,500–500 nm). In opposition to the well-known MIR spectroscopy (4,000–400 cm^{-1} or 25–2.5 µm) which consists of sharp fundamental vibration modes, NIR spectroscopy consists of overtone and combination of vibration modes. The main advantage of these absorption modes is their weak intensity enabling the analysis of the sample without dilution. But their high degree of overlapping implies large absorption bands hindering the visual spectrum interpretation. Therefore, chemometrics are required to extract the useful information of NIR spectra. Another point to mention is the sensibility of this technique to environmental conditions (i.e.,

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temperature, humidity) and the influence of the physical and chemical properties of the samples under investigation.

The largest opportunity for NIR is based on its ability to provide a fast analysis directly on the sample (e.g., tablets, powder) and possibly through the packaging. In addition, the launch of handheld and miniaturized NIR spectrophotometers is another huge opportunity to develop field analysis.

One important threat concerns the lifecycle of NIR calibration model. To develop the calibration of quantitative NIR models, samples must be analyzed by both NIR and a reference method (e.g., LC). Moreover, the samples and environmental conditions (temperature, humidity,.) used during model development must necessarily mimic routine use of the future method to avoid biased results. In addition to this time- consuming phase to establish the quantitative calibration model, the latter should be properly maintained during the routine use of the method. The development and maintenance of NIR calibration model requires resources, chemometrics knowledge and well-trained analysts.

Finally, the transferability of NIR model between instruments may constitute a limitation to implement this technology for quality control labs of solid and liquid samples.

2.2. SWOT ANALYSIS OF RAMAN SPECTROSCOPY [9–13]

Like NIR, the main strengths of Raman spectroscopy originate from those of vibrational spectroscopy: it is a green, fast, and non-destructive technique. Usually, no sample preparation is required and analysis trough the packaging is also possible. Moreover, a small volume of the sample (equivalent to the laser spot) is needed to perform the measurement. Contrarily to NIR spectroscopy, Raman spectra exhibit sharp features making their visual interpretation and sample characterization possible. Raman bands are sensitive to the solid state of the sample enabling the polymorphism tracking (identification and quantification) especially when working in the low frequency (10–400 cm^{-1}) domain. Moreover, the sharpness of Raman bands usually makes it possible to identify and monitor specific compounds using a non-overlapped band. It is important to notice that despite the possibility to monitor one specific band, chemometrics are often used to perform qualitative and quantitative analysis. This technique can be used for the analysis of solids, liquids (solution and suspension), and gas. Finally, Raman spectroscopy being almost insensitive to water is the preferred choice for the analysis of aqueous samples (e.g., solutions, biological media, etc.).

The weaknesses of Raman are mainly related to its low sensitivity. Another main issue to deal with is the alteration of Raman spectrum by fluorescence of the sample. The selection of the wavelength of the laser and therefore the laser source itself is a critical step when setting up experiments. Indeed, low incidental wavelength (e.g. 532 nm) provides high Raman signal intensity but are generally overwhelmed by fluorescence background while high wavelength (e.g. 1064 nm) provides fluorescence-free spectra but the Raman intensity is generally weak leading to long acquisition times. Generally, for pharmaceutical QC purposes, the best compromise between signal intensity and fluorescence interference is found with the 785 nm laser wavelength source. It is important to notice that the sample heating by the focalized laser radiation can degrade or destroy the sample which is especially true for IR laser sources. Regarding the instrumentation itself, this technique requires high-

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stability laser sources and effective system to collect, detect and analyze the weak Raman signal. The instrumentation is quite expensive, but this is counterbalanced by the low-routine analysis cost (speed of data acquisition speed, absence of solvents, reagents, etc.) in comparison with separative techniques.

The possibility to combine Raman spectroscopy with other optical devices could be presented as a major opportunity. For instance, the use of an optical probe could allow to perform online analysis (e.g., follow up of chemical or biological process). The combination of Raman spectrophotometer with optical microscope represents another opportunity allowing Raman hyperspectral image acquisition. To overcome the poor sensitivity of the technique, surface-enhanced Raman spectroscopy (SERS) could be proposed. SERS provides a signal exaltation factor from 10^3 to 10^{14} to reach high sensitivity technique. Nevertheless, even its advantages for several application domains, this nice evolution of Raman spectroscopy remains difficult to properly control for QC quantitative analysis due to its high variability against pharmaceutical requirements.

The main threat of Raman spectroscopy could be the competition with NIR. Its low sensitivity and high-cost instrumentation could be a hindrance to the development of this technology.

2.3. NIR AND RAMAN FOR PHARMACEUTICALS QUALITY CONTROL

Many applications of NIR and Raman spectroscopy for pharmaceutical quality control are described in the literature. Some examples are discussed in the present section to illustrate the interest and potential of these techniques. A summary of discussed applications is presented in Table 1. Thanks to its fast analysis time, NIR is generally implemented for real-time analysis (in-process control). A recent study proposed a spatially resolved NIR instrumentation to enable real-time multipoint measurements [14]. This system was directly embedded into the manufacturing in-line control unit with 3D microwave resonance technology. The combination of both techniques helps to measure the API dosage of each tablet (by means of API content and tablet mass measurement). Another study deals with the challenge of suspension manufacturing real-time NIR analysis [15]. A validated NIR method developed on laboratory scale was applied in the real-time manufacturing control. The predicted NIR assay fluctuates with time while the LC measurements (reference method) do not present this variable profile. The flow turbulence leads to an instable and inconsistent suspension in the front of NIR sensor. The interface was then optimized to control this effect and enable NIR in-line control. This study illustrates the potential of NIR for in-line control, even with tricky applications and demonstrates the interest of proper sensor interface. Raman is also used for in-line manufacturing quality control. A recent study proposed the use of Raman to simultaneously control the API content (ramipril) and its degradation during hot-melt extrusion (HME) [16]. This simultaneous analysis was performed thanks to the monitoring of different spectral regions. Partial Least Squares (PLS) regression model for inline Raman was validated showing the adequacy of the technique, the Raman-HME interface and multivariate analysis. Nevertheless, external validation is required to further develop a quantitative model.

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TABLE 1 NIR AND RAMAN PHARMACEUTICAL APPLICATIONS

Reference	Analytical technique	Application	Compound (s) of interest	Matrix	Method validation
[14]	Spatially resolved NIR spectroscopy	In-line assay for real-time manufacturing control	Diclofenac	Tablets	Predictive model validation
[15]	NIR spectroscopy	In-line assay for real-time manufacturing control	API *(confidential)*	Suspension	Predictive model validation
[16]	Raman spectroscopy	In-line assay for real-time manufacturing control	Ramipril	Extrudates	/
[17]	Handheld NIR and Raman spectroscopy	Falsification detection (API identification)	Ibuprofen, paracetamol, arthemeter- lumefantrine	Tablets, capsules	/
[18]	Raman spectroscopy	Substandard detection	Painkillers (paracetamol, ibuprofen, acetylsalicylic acid)	Tablets	Evaluation of model prediction using random data of calibration set
[19]	NIR and Raman spectroscopy	QC	Paracetamol, acetylsalicylic acid, caffeine	Tablets	NIR and Raman models validation) Precision evaluation: one concentration level
[21]	Transmission Raman spectroscopy and NIR hyperspectral imaging	QC	Caffeine, API A *(confidential)*	Tablets	Model cross validation
[22]	Raman spectroscopy	Hospital preparation QC	Antineoplastic drugs (fluorouracil, gemcitabine, cyclophosphamide, doxorubicin, ifosfamide)	Aqueous solution	Model validation Analytical method validation: accuracy profile
[32]	Handheld NIR spectroscopy	QC Falsification Calibration transfer	Metformine HCl	Film coated tablets	Model validation Analytical method validation: accuracy profile
[33]	Portable NIR spectroscopy	Polymorphs QC Calibration transfer	Mebendazole polymorphs A, B and C	/	Predictive model validation

Next to the in-line control of manufacturing, NIR and Raman are also well-described for the analysis of drug products when a fast analysis is advised. A first study compares the performances of handheld NIR and Raman spectrophotometers for qualitative analysis to detect falsification [17]. The objective was to compare benchtop NIR, low-cost NIR, FDA qualified Raman and middle cost Raman devices. As expected, NIR devices were more sensitive to the physical state of the samples. Raman was strongly influenced by the diffusion of highly dosed API. This phenomenon led to masking the Raman signal of lower doses of poor Raman scattering compound. Even if no formal conclusion about the 'ideal' spectrophotometer could be drawn, several important points were highlighted. For specific product identification purpose, NIR devices are more reliable than Raman. Moreover, handheld NIR devices offer almost comparable identification performances than benchtop. Raman is more interesting for chemical interpretation of the presence, absence, or substitution of API. Another study, published by authorities, proposed a Raman method to detect substandard painkillers [18]. Two subsequent models

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were used, a Soft-Independent Modelling of Class Analogy (SIMCA) model for the API identification and a PLS model for API quantitation. Raman associated to chemometrics is presented as a rapid tool to verify the presence and the declared amount of API (i.e. nominal amount of API between 15 % and 85 % w/w). This system could be used in customs or for forensic analysis. Despite the real interest of this approach, it is important to notice that the method was not validated. Even if a proper method validation is not mandatory for this application, model validation using an independent data is clearly advised. In this context, Pino-Torres et al. proposed a fair comparison of NIR and Raman to quantify API in solid formulation [19]. Using a model formulation composed of 3 API (paracetamol, acetylsalicylic acid and caffeine), they proposed a suitable methodology to build the calibration set. It includes different API concentrations, different excipients contents and commercial samples to include some physical variables of the manufacturing process. Building the calibration set is indeed a critical step to obtain quality prediction results and then provides solid foundation for a suitable analytical method. The validation set includes laboratory-made and commercial tablets. Moreover, a validated HPLC method was used as reference method. Usually, it is commonly admitted that NIR requires the creation of a calibration model that should be validated using an external validation set. This common approach was properly used in the referenced study to select the calibration model enabling suitable prediction (generally evaluated by RMSEP criterion). Model validation should not be confused with analytical method validation, which is the proper evaluation of method quantitative performances [7,20]. Here, repeatability and intermediate precision were estimated with suitable RSD (%) values (< 2 % for NIR and < 3 % for Raman). NIR method presents better precision than Raman. Nevertheless, this estimation was performed for only one concentration level (target concentration in routine analysis) using the same tablet for measurement repetitions. This strategy is obviously not representative of routine use of the method and could lead to underestimation of method variability. To conclude this study, it appears that NIR is a simpler and faster technique for quantitative analysis while Raman should be preferred to evaluate API distribution in the tablets. In the same way, another team reported the use of NIR hyperspectral imaging and Raman spectroscopy in transmission mode to perform uniformity content analysis of tablets [21]. In addition to the usual API concentration range to build the calibration set, the tablets were manufactured using different compression and compaction forces to mimic API concentration gradient. Model cross-validation was performed for both techniques: Raman methods RMSEP was lower than NIR, highlighting better predictions. This could be explained by the signal measurement nature: the scattering is collected for tablet core with transmission Raman while only the surface information was measured for NIR. These analytical techniques could outperform usual HPLC analysis thanks to a relevant decrease of analysis time for uniformity of content. Nevertheless, in routine analysis, this latter is generally required for low-dosed API. An evaluation of NIR and Raman using more relevant model samples is required.

Model building and validation remains a critical step to implement NIR or Raman methodology. In this context, an in-depth strategy was proposed for the identification and the quantification of antineoplatic drugs [22]. Drug discrimination (i.e., identification) was performed using PLS-DA model before performing the quantitation. As usual, a calibration set was used to build the model and the validation set was used to optimize and validate the model. Afterwards, an external validation set, including independent samples, was used to evaluate the predictive performances of the validation model. This last step could be assumed as method validation. Indeed, independent samples were

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analyzed using the optimized method and appropriate calibration model. Validation strategy using accuracy profile helps to estimate all validation criteria required by ICH Q2 (R1) and is now advised by USP [23–28]. Such approach should be also adopted soon by ICH with the currently under revision ICH Q2 (R2) [29]. Performing a complete analytical method validation is required to assess its performances for pharmaceutical quality control. It is important to notice that acceptance limits for method validation were set at ± 15 %, which is not in accordance with DP release but could be acceptable for hospital preparations described in the paper.

Next to method validation, the method and model transfer is another important step. It relies on the transfer of a validated method from the sending unit to one or several receiving units. For NIR methods, the challenge consists of the transfer of calibration model developed on one specific data set and under stable environmental conditions. Several approaches are well-described in related literature [30,31]. A recent study investigates different calibration-transfer strategies using low-cost handheld NIR devices [32]. The first step was to develop and validate the PLS model for the determination of metformin using handheld NIR device. Several chemometrics strategies help to manage the spectral variations between instruments by correction or removing the spectral drift. Using a global model strategy (i.e., a PLS model based on calibration data of all NIR devices), the different sources of variability are considered, which allows to propose a robust model. The lack of instrumental information and the quite low number of instruments involved are limitative to draw formal conclusion. NIR transfer remains a tricky and challenging step while the demonstration of method transfers and reproducibility evaluation is required for large-scale QC analysis and mandatory to establish normative methods (i.e., Pharmacopeia method). Another project was focused of the quality control of polymorphs in pharmaceutical raw material using NIR with an investigation of calibration transfer from benchtop to portable instruments [33]. PLS model adapted to each instrument was proposed to evaluate polymorphs composition of mebendazole raw material. Furthermore, calibration model transfer was investigated with satisfactory results. This study highlights a flexible, easy, cheap, and fast way to control polymorphism of incoming material for pharmaceutical manufacturing. Despite its relevant topic, this study deserves a deeper investigation with an independent testing set for calibration models and transfer assessment. These interesting publications highlight the growing interest for NIR and Raman spectroscopies for fast and in-situ analysis and for polymorphs control. Both aspects are really challenging and relevant in the context of pharmaceutical quality control. Another important aspect to stress out is the capital importance of NIR and Raman sensors as Process Analytical Technologies for the implementation of a control strategy in the framework of continuous manufacturing processes and real-time release testing [34,35].

Separation techniques

Next to vibrational spectroscopic techniques, separation sciences remain up to now the predominant analytical tool used for QC analysis and are clearly preferred for the analysis of complex samples and/or low-dosed compounds. Among them, two techniques knew an increase of interest since the 2010′s. Supercritical fluid chromatography (SFC) and 2-D chromatography are discussed in the present section.

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As done previously for spectroscopic techniques, SWOT analysis and relevant applications are presented and discussed.

SWOT ANALYSIS OF SFC [36–42]

The core strength of SFC relies on the use of supercritical CO_2 mobile phase. Thanks to the supercritical fluid properties, the possibility to operate at high flow rate and/or to use sub 2-µm particles under conventional pressures (< 600 bars) without loss of efficiency or resolution are the main strengths of SFC. The drastic reduction of solvent use in comparison with LC offers also significant advantages regarding the analysis cost and environmental impact. Next to these assets of SFC, recent publications also highlight the easy and robust hyphenation with MS thanks to the availability of dedicated interfaces. Finally, the versatility of SFC is more and more investigated thanks to (i) the possibility to adjust the mobile phase elution strength by the pressure, the temperature, or the organic modifier content and (ii) the miscibility of CO_2 with organic solvents in relative high proportion extending the polarity of target analytes.

One main SFC weakness is related to the instrumentation. Despite the recent technological improvement with the launch of modern instruments, the extra-column band broadening is still significantly higher compared to UHPLC instruments. Extra-column variances of 70 – 100 μL^2 vs 1–2 μL^2 are reported for state-of-the-art SFC and UHPLC instruments, respectively. This drawback directly impacts the chromatographic performances when efficient columns with reduced dimensions and small particles are used. Moreover, despite in-depth fundamental studies of SFC method transfer, the instrumentation transfer remains challenging. Another historical weakness of SFC is its relative low UV-sensitivity. Technical improvements, especially related to the detection cell, helped to reduce the sensitivity difference between SFC and LC. Nevertheless, the limit of detection of LC-UV methods generally remains lower compared to SFC-UV methods. Nevertheless, this lower UV sensitivity is easily compensated by SFC hyphenation with MS. Finally, the new application fields of SFC brings new challenges. Indeed, the shift from classical application of SFC for the analysis of small apolar molecules to larger and more polar molecules leads to the use of mobile phase compositions with viscosities close to those used in LC, reducing the kinetic performances of SFC. In these conditions, the higher operating pressure of the instrumentation becomes too restrictive preventing the use of high flow rates and sub-2-µm particles.

Ten years ago, during SFC resurgence, the main opportunity for this technique was the possibility to totally replace normal phase LC. Based on recent developments and applications, the actual major opportunity of SFC is to replace several other LC modes thanks to its versatility. The ultimate goal being the separation of both non-polar and polar analytes within one run, using a large gradient. This SFC-related technique named unified chromatography aims to combine different separation modes using a large gradient from 0 or 2 to 100 % of modifier in CO_2. Another advantage is the possibility to use on-line supercritical fluid extraction (SFE) as sample preparation technique. This direct hyphenation aims to ease sample preparation and improve extraction recovery and reliability. The main threat of SFC is its poor implementation in routine laboratories. Despite a large scientific interest during the last decade, the technique seems to get stuck in R&D laboratories while systems in normal phase are maintained by habit, fear of change or lack of expertise within QC laboratories. A direct negative impact

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on instrumentation development may be feared since the analytical instrument manufacturers will likely focus their R&D budgets on more profitable technologies.

SFC FOR PHARMACEUTICAL QUALITY CONTROL

The interest of SFC for pharmaceutical quality control is now well- described in the literature [43]. A summary of discussed SFC applications is reported in Table 2.

TABLE 2 SFC PHARMACEUTICAL APPLICATIONS

Reference	Application	Compound (s) of interest	Matrix	Method validation
[44]	Enantiopurity	Pharmaceuticals (e.g. cetirizine, epinephrine, ezetimide, ibuprofen, ketoprofen, maraviroc, tamsulosin, zolmitriptan, etc.)	/	/
[45]	Chiral separation	Multiple chiral centers compound (dihydropyridone derivative)	/	/
[46]	Enantiomeric excess determination during enantioselective synthesis	Verubecestat	/	/
[47]	Nitrosamines	Nitrosamines in sartans	DS and DP	Specificity and LOD
[48]	Nitrosamines	Nitrosamines in ranitidine	/	Robustness
[50]	Drug and counter-ion stoichiometry	Ondansetron hydrochloride	/	Full validation according to total error approach
[51]	Polar compounds separation using new mixed-mode stationary phases	Amines (e.g. serotonin, propranolol), zwitterions (e.g. tryphtophan), amides (e.g. melatonin)	/	/
[53]	Inhaler quality control	Ciclesonide and impurities	Metered-dose inhaler	/
[54]	Impurities QC	Aglometine, atorvastatine, enzalutamide, ticagrelor impurities	/	Full validation according to ICH
[55–57]	Impurities QC	Salbutamol impurities	/	Full validation according to total error approach Reproducibility (inter-laboratory studies)

Historically, one of the main applications areas of SFC was chiral separation. In this context, several studies consisted in proposing generic SFC conditions for chiral screening. Indeed, it is well-known that the biological and pharmacological properties differ between enantiomers. Enantiomeric purity is therefore required for drugs presenting a potentially toxic enantiomer. This assay could also be required if a pure enantiomer is marketed to avoid potential side-effects of non-active enantiomer. In this context, when the enantiopurity control is performed, the typical authorized limit for a chiral impurity is around 0.1–0.2 % relative to the API. It results in chromatograms with a very wide peak of API often leading to (strong) peak tailing. An in-depth study is proposed by Novakova et al. [44]. Using 20 model chiral compounds with different chemical properties, several stationary phases and mobile phase compositions were tested with a generic gradient method. This study highlighted that the use of combined additives (i.e., DEA and TFA) provided a high success rate of enantiomeric separation in comparison with usual single additive. Around 60 % of enantiomeric pairs were successfully separated during this screening phase. Afterwards, they applied this strategy to optimize real-life separation. For screening separations exhibiting a resolution lower than 2 between the API and the enantiomeric impurity, a further optimization was performed. Indeed, a higher resolution was required to anticipate

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real sample applications because of the concentration ratios of both enantiomers. Column temperature, pressure, gradient slope, and additive concentrations were optimized in this regard. As illustrated in Fig. 1, method optimization improved enantioseparation. The increase of DEA concentration was, in this case, useful to the method selectivity and inverse elution order. The main drawback of this study is the incompatibility of mobile phase additives with MS detection Further work is required to propose alternative additive while keeping suitable enantioselectivity.

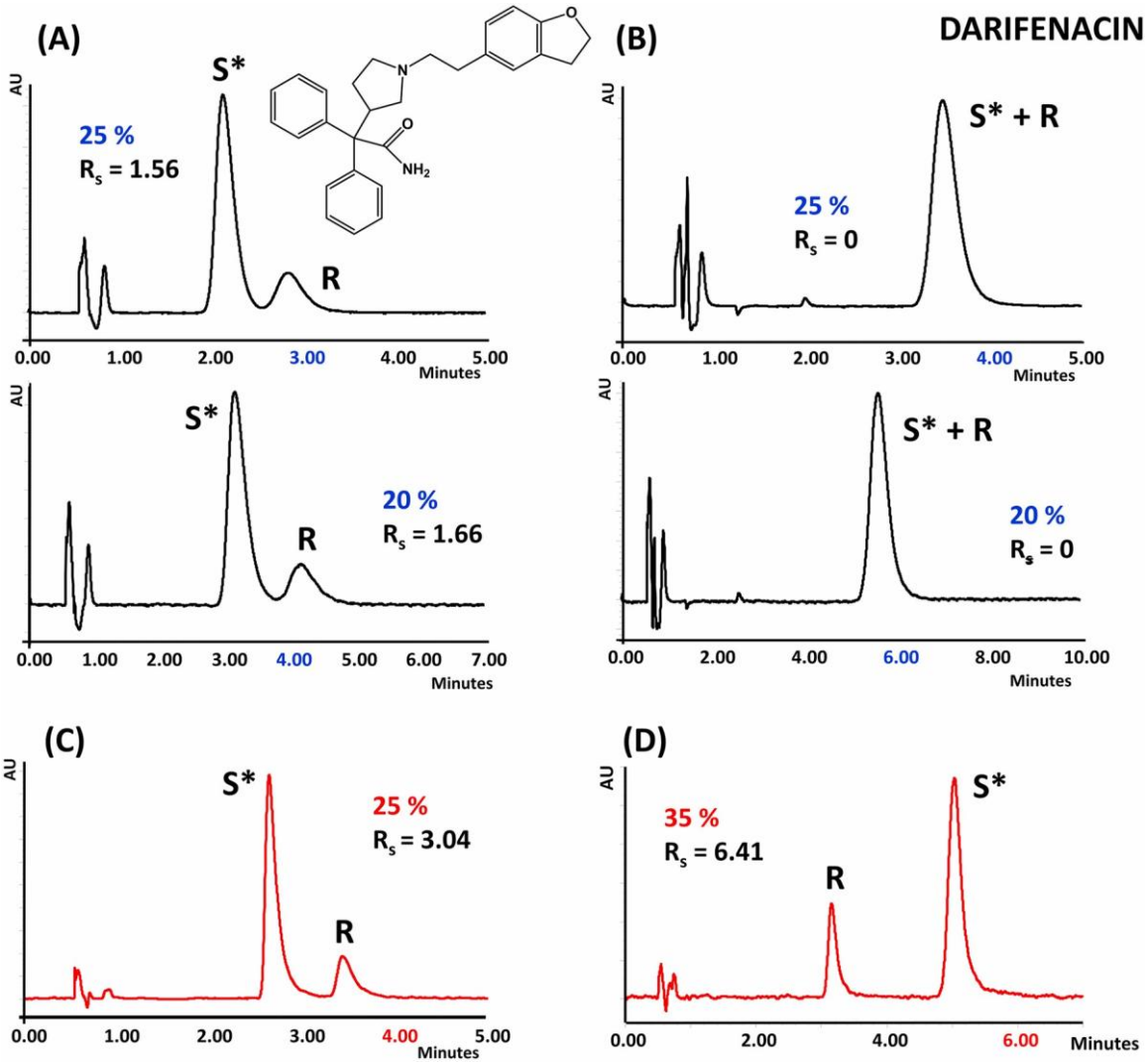

Fig. 1. SFC enantioseparation of darifenacin on Amycoat column – study of organic modifier amount and additive concentration effects on the enantioseparation. (A) CO_2/methanol with 0.05 % TFA and 0.05 % DEA (B) CO_2/methanol with 0.05 % TFA and 0.5 % DEA (C) CO_2/isopropanol with 0.05 % TFA and 0.05 % DEA (D) CO_2/ isopropanol with 0.05 % TFA 0.5 % DEA. The active isomer is depicted with asterisk.

Another research group proposed the evaluation and comparison of three LC modes (normal phase (NPLC), polar aqueous-organic mode and polar organic mode and SFC for the separation of eight stereoisomers of the dihydropyridone derivative 1 containing three chiral centers [45]. The final objective of this study was to propose a quality control method to assess chiral purity for individual stereoisomer during pharmacological assay. Complete separation was reached using optimized SFC method with two-columns coupled in series. This approach improved resolution and efficiency at the

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cost of increased analysis time. In LC, only the normal phase mode was successful. Regarding the environmental impact, SFC is clearly greener than n-heptane based mobile phase. Another strategy to improve the generally lower efficiency of chiral separation, superficially porous particles were used for the follow-up of enantioselective pharmaceutical synthesis [46]. This particles technology was used for SFC and LC chiral separation and allows highly efficient enantio-separation. Despite the high interest of SFC of chiral separation, the evaluation of method quantitative performances is still poorly reported in the literature.

Next to the traditional NPLC alternative applications, SFC is increasingly used for unusual applications. A current hot topic in the pharmaceutical industry concerns nitrosamines impurities since they are classified as probable human carcinogens. Regulatory agencies (EMA and FDA) asked all marketing authorisation holders to conduct a review of their products on the potential risk of containing nitrosamine impurities. The watchlist of nitrosamines is continuously completed based on investigations progress. In this context, Schmidtsdorff et al. [47,48] proposed SFC-MS/MS method for the detection of nitrosamines in drug substances and drug products. The methods were validated for limit testing in accordance with ICH Q2 (R1) with limit of detection in the ng/mL order corresponding to less than 1 ppm in the medicine tablet. In the second study, sample analysis uncovered nitrosamines contaminations in ranitidine samples at 10-times above the tolerance limit. These studies highlight the readiness of SFC to meet current challenges in the pharmaceutical industry, as stand-alone or complementary to usual techniques (LC/GC).

A second original application is the coupling of evaporative light scattering detection with SFC which was successfully used to determine simultaneously inorganic anions and cations (Mg^{2+}, Li^{+}; Cl^{-}, Br^{-}, NO^{-}_{3}, I^{-}, SCN^{-}) [49]. This significant contribution proposes optimized SFC conditions to analyse inorganic ions in aqueous samples. This approach was used to determine the stoichiometry of a drug and its counter-ion (ondansetron hydrochloride) in a single SFC method using UV and ELSD as illustrated in Fig. 2 [50]. To the best of our knowledge, the simultaneous (in a single run) determination of a drug and its counter-ion could not be easily performed by any other separation techniques. The method was validated following the total error methodology.

Another work was focused on mixed-mode stationary phase synthesis and evaluation to enhance the selectivity of chromatography for polar compounds [51]. High retention was reached using ion-exchange-mixed-mode stationary phase in SFC with an accurate overlay of 15 injections demonstrating the retention time repeatability. Using gradient elution, compounds were separated in 10 min. In the same way, recent publications highlight the versatility of SFC with the separation of strong polar compounds like metabolites or biomolecules [38,52]. The online hyphenation of SFC with extraction process is also a major advantage of this separation technique. In this context, SFE-SFC-MS method is proposed to analyse simultaneously ciclesonide and its impurities in inhaler [53]. This technique helped to directly transfer the extracted sample to SFC for quality control. More data are required to evaluate the extraction yield and other quantitative performances of the method.

It is important to remind that SFC was previously associated with poor performances regarding repeatability and robustness. These drawbacks were mainly related to the former SFC instrumentation. As modern SFC instrumentation was introduced during the last decade in the analytical portfolio, several studies were performed to evaluate and demonstrate the quantitative performances of this

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DOI: 10.1016/j.jpba.2022.115071
Status : Postprint (Author's version)

technique. In this context, Plachka et al. ´ [54] proposed SFC for the determination of impurities in 10 pharmaceutical mixtures. The validation design permitted to assess all the required validation criteria of ICH Q2 (R1), including LOD (< 2 ug/mL) and LOQ (1–10 ug/mL) for impurities. Next to the usual validation criteria, it is interesting to evaluate the third and highest level of precision, i.e., reproducibility. From this perspective, SFC quantitative performances were deeply evaluated by means of (i) total error approach validation, (ii) inter-laboratory study using one type of SFC instrument and (iii) multi-systems inter-laboratory study [55–57]. During the two first steps, the precision of the method was estimated in terms of repeatability (between 2.8 % and 3.4 %) and reproducibility (between 7.7 % and 10.7 %). These results highlighted the excellent quantitative performance of SFC and demonstrated the technique to be on par with other chromatographic techniques. In the second interlaboratory study, the extension to multi-systems comparison led to reproducibility RSD (%) values between 15 % and 17 % which remains excellent considering the multiple sources of variability. These studies demonstrated that SFC could be implemented in multiple laboratories working with different instruments. Nevertheless, SFC method transfer between instruments remains challenging and deserves deeper investigations.

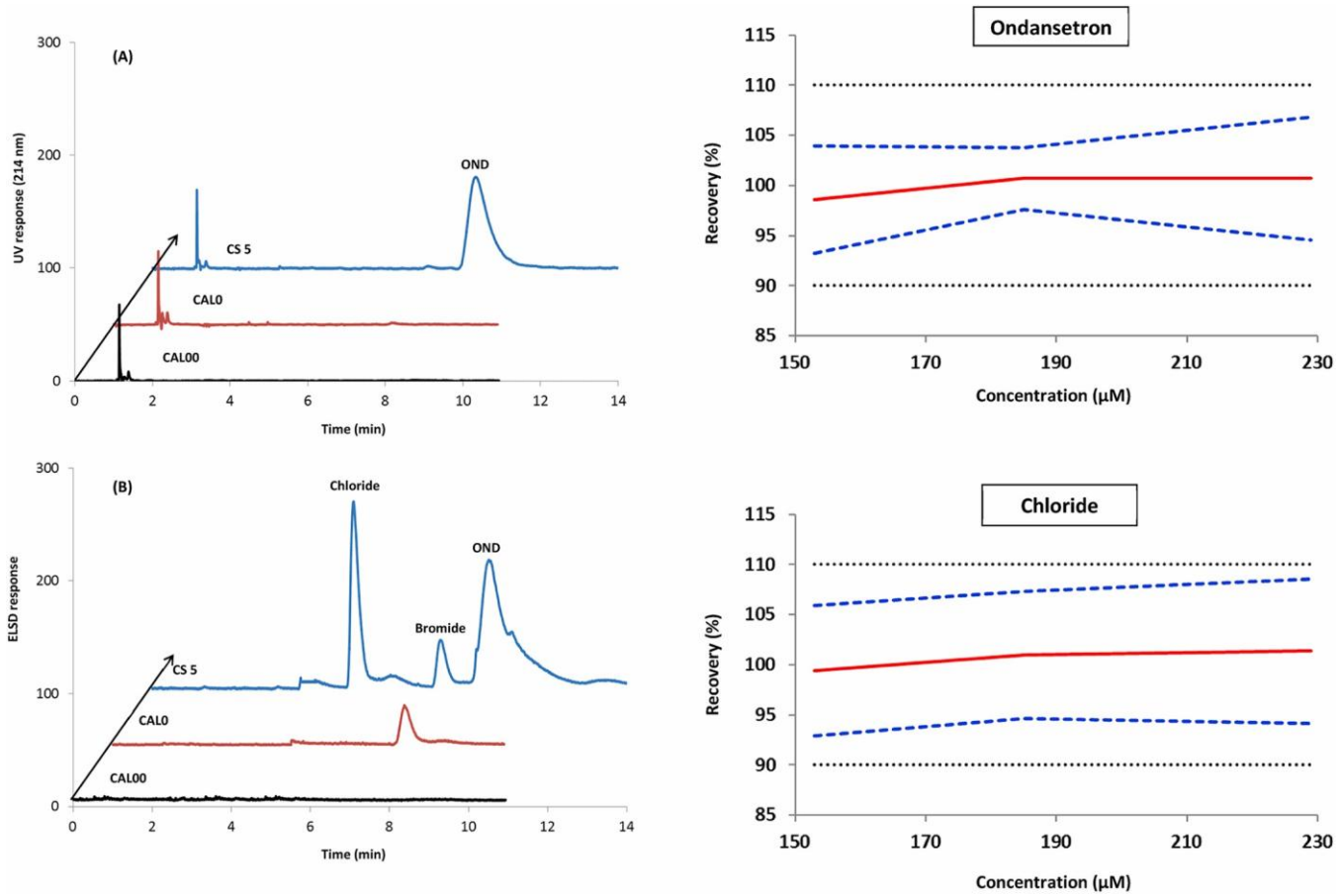

Fig. 2. SFC-UV-ELSD isocratic separation of ondansetron chloride using 1-ethylpyridine stationary phase and CO_2 with 12 % of co-solvent (MeOH/H_2O/DEA – 98/2/ 0.2 – v/v/v) as mobile phase. Temperature 35 °C, pressure 150 bar, flowrate 3 mL/min. (Left) (A) SFC-UV and (B) SFC-ELSD chromatograms of a blank sample (CAL00), a blank sample spiked with scopolamine bromide to assess method selectivity (scopolamine peak) (CAL0) and a calibration standard (CS5). Section 2 CAL0: [Bromide] = 65 µM; CS5: [Ondansetron] = [Chloride] = 229 µM, [Bromide] = 65 µM. Bormide was used as internal standard. (Right) Accuracy profiles for the analysis of ondansetron by SFC-UV and chloride ion by SFC-ELSD using linear regression. The black lines represent the acceptance limits (90–110 %), the red line corresponds to the recovery and the confidence interval of the recovery at a risk of 5 % is materialized by blue lines.

Published in : Journal of Pharmaceutical and Biomedical Analysis (2022), 221, p. 115071
DOI: 10.1016/j.jpba.2022.115071
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SWOT ANALYSIS OF 2D-CHROMATOGRAPHY [58–62]

According to Giddings, the terminology "multi-dimensional separation" refers to a technique in which: (i) components are subjected to two or more largely independent separative displacements and (ii) components adequately resolved in the first separation dimension, remain resolved throughout the whole separation process. 2D-chromatography can be carried out in off-line or on-line mode. Off-line mode consists of a fraction collection from the first dimension for a reinjection into a second dimension later. In opposite, on-line mode consists of the use of dedicated instrument to automatically perform the transfer from the first to the second dimension.

The strengths of 2-D chromatography rely on the variability of possible combinations of selectivity of both dimensions using orthogonal separation modes or techniques. It means that, for example, RPLC in the first dimension could be combined with HILIC in the second dimension or with SFC. These infinite combinations of chromatographic selectivity represent a unique opportunity of separation for complex samples. Moreover, depending on the requirements, two main approaches are available. (i) Heart-cutting approach (e.g., LC-LC) consists of transferring only a limited portion of sample from the first dimension (most often a unique fraction) to the second dimension. This approach is used when only a few compounds of the sample are of interest. (ii) Comprehensive approach (e.g., LC×LC) consists of transferring the entire sample in both dimensions. Successive fractions of the first dimension are then stored and successively analysed in the second dimension. An alternative approach, named the selective comprehensive mode, consists of sampling selected regions of the first dimension for the second-dimension separation. It means that the two-dimensions analysis could be properly adapted to the sample and analysis requirements.

Regarding the weaknesses of multi-dimensional chromatography, two main points could be highlighted. Firstly, method development is more complex and time-consuming than 1D-separation. Several parameters should be properly set and considered like solvents compatibility. Secondly, the instrumentation setup and separation complexity are correlated with higher variability than usual 1D separation.

Recent pharmaceutical compound development and regulatory requirements evolution lead to the need of more and more powerful analytical technique. Identifying impurities with closely related structure and sometimes unknown impurities is challenging even with modern analytical technique. In this context, the evolution of pharmaceutics (and other applications area) represents a major opportunity for multi-dimensional separation and especially 2D-chromatography. For now, the main threat of 2D-chromatography is related to the instrumentation. Despite the availability of 2D-LC instruments, the use of such systems remains complex and challenging to integrate in routine laboratory. Moreover, LC×SFC instrumentations are yet developed in laboratories without the launch of commercial instrumentation. Such equipment could not be integrated in routine laboratory.

MULTI-DIMENSIONAL CHROMATOGRAPHY FOR PHARMACEUTICAL QUALITY CONTROL

As expected, multi-dimensional chromatography is mainly reported for the analysis of complex samples (Table 3.). In this context, the determination of pharmaceutical impurities is deeply reported

Published in : Journal of Pharmaceutical and Biomedical Analysis (2022), 221, p. 115071
DOI: 10.1016/j.jpba.2022.115071
Status : Postprint (Author's version)

in the literature. A first study investigated comprehensive LC×LC to propose generic 2D systems for the analysis of API and related synthesis intermediates [63]. Method development was focused on maximizing the separation power and reaching sufficient orthogonality between both dimensions. Optimized systems using RPLC in both dimensions were evaluated using two model sample mixtures (confidential information). Peak capacity close to 1000 with analysis time of 50 min highlight the relevance of optimized 2D-systems. In comparison with LC, the use of LC×LC significantly increased the number of detected peaks.

The selection of suitable and orthogonal stationary phases is the starting point of 2D method development. Therefore, the automatized screening of multiple columns in both dimensions and mobile phases was evaluated [64]. To highlight the relevance of 2D separation, pharmaceutical reaction mixtures where traditional 1D RPLC and SFC fail or deliver suboptimal results were selected as case study. For this application, the first dimension aimed to separate target compounds, including starting materials and other by-products from the chemistry process while a chiral or achiral separation is used in the second dimension to separate unresolved peaks. This instrument helped to automate the screening phase and speed up the whole analytical process to reach an efficient method. The automated multicolumn online 2D-LC-DAD-ESI-MS instrument was successfully used to separate and analyse complex mixtures containing chiral and achiral pharmaceuticals. The repeatability of retention was estimated for both dimensions: RSD (%) lower than 3 % and 0.5 % were measured for the first and the second dimension respectively. These values highlight the repeatability of the 2D separation process. To ensure orthogonality between both dimensions, another study proposes the use of temperature responsive LC (TRLC) in the first dimension [65]. It consists of using as stationary phase a smart polymer whose properties change due to an environmental stimulus. Here, it led to hydrophobic retention for temperature above 32 °C while a decrease of retention is observed for lower temperature. The use of this original combination of retention mechanism helped to successfully separate impurities from the overloaded API signal. Moreover, RT and peak volume precision were estimated with suitable RSD (%) values (RSD > 0.7 % for RT and < 1.5 % for peak volume). Despite these interesting results, the use of laboratory made TRLC column represents the main limitation of this system.

2D-LC separations were successfully used for dedicated pharmaceutical applications. Selective comprehensive SEC×RPLC was proposed to detect oligomeric impurities [66]. The presence of these oligomeric impurities in small molecule drugs is indeed overlooked because most of the RPLC methods used in small molecules QC could not separate the oligomers. The coelution of SEC peaks were resolved in RPLC highlighting the orthogonality between both dimensions. Moreover, the consistency of first dimension SEC profile was demonstrated with retention time RSD values lower than 0.4 %. Finally, 2D-LC was hyphenated with Q-Exactive MS for oligomeric impurities structure identification. This study underlines the complementarity between analytical tools, especially for challenging analysis. 2D-separation is also helpful for the quality control of new therapeutic molecules such as siRNA [67]. Using anion-exchange in the first dimension and MS-compatible ion-pairing RPLC in the second dimension allowed the detection of the UV-trace of numerous impurities (at concentration in the pg/mL order). Another study proposed the used of mixed-mode and RPLC 2D separation for the characterization of oligonucleotides [68]. Multi-dimensional chromatography is also an attractive tool for the quality control of large molecules such as peptides or proteins [69], but it is out of the scope of the present discussion.

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In addition to the demonstration of separation and detection capability of 2D-LC, it is also mandatory to evaluate to the quantitative performances of the technique. This was investigated by Iguiniz et al. [70] for pharmaceutical applications of on-line comprehensive two-dimensional liquid chromatography. Data processing in LC×LC could be performed using three different strategies: (i) summing the successive second dimension peak areas related to the same first peak dimension; (ii) calculating the volume of the 3D peak by considering it as a cone; (iii) considering peak area of the main fraction. Data processing strategies were evaluated using parabens as model compounds. The strategy based on the sum of peak areas is more reliable and avoid under or overestimation. Another aspect to deal with is the dilution effect between both dimensions. Column length and flow rate have a direct impact on peak capacity and on dilution factor. Optimization of these parameters are important to guarantee method sensitivity. Using optimized conditions and selected data processing strategy, method performances were evaluated. Retention times and the sum of peak areas repeatability were estimated with RSD values lower than 1 % and 1.5 %, respectively. Moreover, signal linearity, LOD and LOQ were evaluated according to ICH Q2 (R1). LOD below 0.04 % (% w/w with API) was estimated, this value is in accordance with ICH requirements for the detection of impurities. Another publication reports a systematic validation of a heart-cutting 2D-LC method for pharmaceutical QC release and stability testing [71]. Method validation according to ICH Q2 (R1) was performed for peak integration and quantitation from the 2D method. LOD and LOQ were estimated using S/N at 0.02 % and 0.05 % w/w, respectively. The API was spiked with regiosiomer impurity at three concentration levels to determine method accuracy and precision. Recoveries from 96 % to 127 % were measured with an RSD of 4 % at the LOQ and lower than 1 % for a concentration of 10 % of spiked impurity. Intermediate precision was also evaluated by means of day-to-day, analyst-to-analyst and instrument-to-instrument effects. Furthermore, method robustness was tested for parameters difficult to control in routine. This study demonstrates the suitability of implementing 2D-LC in QC labs from a technical perspective.

Next to the coupling of two orthogonal LC dimensions, multidimensional chromatography could involve different chromatographic techniques. Indeed, SFC could be selected as second dimension separation to propose an orthogonal technique to RPLC. Moreover, NPLC and RPLC are difficult to couple because of mobile phase compatibility, SFC is then generally preferred as second dimension for chiral separation of unresolved first dimensions peaks. Finally, as the second dimension requires fast analysis, SFC is, by definition, a suitable alternative thanks to its high flow rate and kinetic properties. Venkatramani et al. proposed an innovative LC×SFC with a trapping column between both dimensions [72]. The hydro-organic elution fractions from the first RPLC dimension are trapped on a small C18 trapping column. Afterwards, the trapping column is backflushed to send the trapped analytes to the second dimension (SFC column). The volume of the trapping column and retention capacity should be carefully optimized because of its impact on keeping the first-dimension separation. This system was successfully used for the analyse of multiple chiral centres drug substance: the 4 diastereoisomers were separated in the first LC dimension while the enantiomeric pair resolution was achieved in the second SFC dimension. The detection limit of the undesired enantiomer was estimated to be 0.1 %. In the same way, another study proposed a selective comprehensive LC×SFC approach to maintain in the second dimension the resolution achieved in the first dimension [73]. A laboratory-made multiple heart cutting system with multiple sample loops for peak transfer is proposed to hyphenate LC to SFC.

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The sample rate was optimized to reach 4–5 fractions for each peak. This paper also highlights some relevant technical parameters and settings to successfully combine these chromatographic techniques. The reader is kindly invited to read the publication for more information. As illustrated in Fig. 3, this analytical setup helped to simultaneously performed the chemical purity in RPLC (^{1}D) and the enantiomeric purity in SFC (^{2}D). It is important to notice that the chiral separation in ^{2}D-SFC is performed within a few seconds. In comparison with unidimensional SFC, sLC×SFC presents a lower sensitivity (factor around 3) because of extra-column dispersion due to instrumental complexity and sample dilution (^{1}D peak divided in different fractions). Nevertheless, it means that at least 1 % of the undesired enantiomer could be easily detected with sLC×SFC.

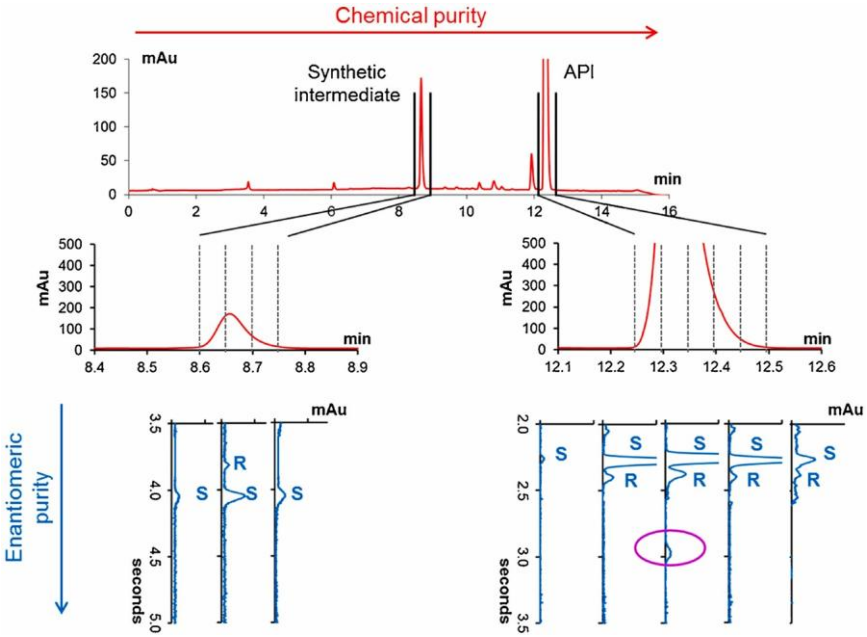

Fig. 3. Achiral-chiral analysis of a pharmaceutical sample by sRPLCxSFC with gradient elution in 2D-SFC: 1D-RPLC chromatogram for chemical purity (top); zoom on chiral compound peaks (middle) and 2D-SFC chromatograms of the different consecutive fractions for enantiomeric purity (bottom). An impurity separated from API in SFC is circled in pink. Reprint with permission from [73].

As described previously, multi-dimensional chromatography is used during chemical synthesis follow-up. To identify potential anomalies in the product synthesis process, the separation of as many positional isomers as possible could be required. In this context, a study compared LC×LC and LC×SFC to analyse a complex pharmaceutical sample containing only neutral compounds but numerous positional isomers [74]. RPLC×RPLC was not really adapted to the separation of all isomeric compounds present in the reaction medium, even a rather good orthogonality between the two dimensions. As expected, HILIC×RPLC presents a poor retention of neutral compounds in the first dimension. RPLC×SFC using a polar stationary phase presents a high degree of orthogonality. High-resolution MS was used as third dimension to differentiate the co-eluted compounds based on their *m/z* ratio. SFC could also be used in the two dimensions [75]. Achiral SFC in the first dimension and chiral SFC in the second dimension with UV and MS detection were successfully used for enantiomeric purity analysis. Using a laboratory-made device, online fraction transfer was performed using loop. Such technology is useful for the enantiomeric analysis and separation of pharmaceutical racemates. The interest of SFC as an

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DOI: 10.1016/j.jpba.2022.115071
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alternative to LC or even more RPLC was previously discussed. In the same way, achiral SFC × chiral SFC could be an alternative to the coupling of RPLC and chiral SFC.

Multidimensional chromatography provides a relevant improvement of method selectivity and separation capabilities. The interest of such coupling was demonstrated for several applications. More dedicated work focused on quantitative aspects is required to extent the interest of these techniques for formal pharmaceutical quality control.

Conclusion

Emerging analytical techniques help to face a large panel of analytical challenges: from fast in-situ API quantitation using portable NIR instrument to complex multi-dimensional chromatography for impurities profiling. The strengths of these technologies are mainly correlated to their emerging character. The recent innovations regarding the instrumentations led to the availability of state-of-the-art equipment with optimal performances. Moreover, the decrease of solvent use and analysis time, and the limitation of analytical techniques required for complex sample analysis helped to minimize ecological footprint of QC analysis. Furthermore, emerging technology means academia interest to deeply study and optimize their performances and possibilities. The publications related to these techniques is expected to increase in the future to complete the documentation of their applications domain and quantitative performances. It is important to notice that next to usually reported feasibility studies, formal evaluation of quantitative performances by means of method validation are required to convince QC laboratories.

Indeed, the last weak point of the described techniques remain their quantitative aspects before their implementation in routine analysis. For vibrational spectroscopy, even if method validation is well documented, the method transfer (and especially calibration model transfer for NIR) should be deeply considered because of its relevance for pharmaceutical industry. Regarding SFC, the intrinsic performances of different instruments (e.g. injection precision, gradient and pression regulation and sensitivity) remain the main barrier for easy method transfer. As previously discussed, multi-dimensional chromatography requires deeper investigation of its quantitative performances, including the data processing part. Such studies must be carried out to enable the implementation of this technique in pharma QC laboratories. Furthermore, wider implementation of 2D-LC methods for routine use in regulated environment also depends on instrument cost, level of expertise and availability of the instruments across different labs. To remove these barriers, more commercialized standard 2D-LC instruments and user- friendly software for instrument operation and data analysis are as critical as the technical suitability itself. Finally, it is important to remind that no commercial LC×SFC instrument is currently offered. The availability of marketed and qualified instrument and software is mandatory to consider further development of this technology.

Considering the analytical challenges to face regarding method efficiency, fastness, greenness and sensitivity, opportunities for emerging analytical techniques are endless. Furthermore, the constant development of new drugs with their associated QC methods is a major opportunity to develop and propose up-to-date technologies. However, pharmaceutical quality control domain remains

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Status : Postprint (Author's version)

conservative, generally to ensure the respect of all its specific requirements. In this context, the implementation of new techniques is traditionally performed several years after the demonstration of their usefulness in academia and R&D laboratories. The usual selection of gold-standard LC and GC for QC analysis appears as the main threat to the implementation of modern and emerging technologies, whatever their major analytical advantages. With respect to this literature review, it seems obvious that emerging techniques (NIR, Raman, SFC, multi-dimensional chromatography) will be important contributors to the current, next-future and future QC analytical technology panel. Their large implementation in regulated laboratories could be considered as the challenge of the next decade.

CRediT authorship contribution statement

Amandine Dispas: Investigation, Writing – original draft. **Pierre- Yves Sacre:** ´ Writing – original draft. **Eric Ziemons:** ´ Writing – review & editing, Funding **Declaration of Competing Interest**

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Amandine DISPAS reports financial support was provided by European Commission. Amandine DISPAS reports financial support was provided by Walloon Public Service.

Data Availability no data because of review paper.

Acknowledgments

Research grants from the Walloon Region of Belgium and EU Commission (project FEDER-PHARE) to A. DISPAS are gratefully acknowledged.

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