Review

Analytical Chemistry in the 21st Century: Challenges, Solutions, and Future Perspectives of Complex Matrices Quantitative Analyses in Biological/Clinical Field

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Abstract: Nowadays, the challenges in analytical chemistry, and mostly in quantitative analysis, include the development and validation of new materials, strategies and procedures to meet the growing need for rapid, sensitive, selective and green methods. In this context, considering the constantly updated International Guidelines, constant innovation is mandatory both in the pre-treatment procedures and in the instrumental configurations to obtain reliable, true, and reproducible information. In this context, additionally to the classic plasma (or serum) matrices, biopsies, whole blood, and urine have seen an increase in the works that also consider *non-conventional* matrices. Obviously, all these studies have shown that there is a correlation between the blood levels and those found in the new matrix, in order to be able to correlate and compare the results in a robust way and reduce any bias problems. This review provides an update of the most recent developments currently in use in the sample pre-treatment and instrument configurations in the biological/clinical fields. Furthermore, the review concludes with a series of considerations regarding the role and future developments of Analytical Chemistry in light of the forthcoming challenges and new goals to be achieved.

Keywords: method validation; hyphenated instrument configurations; extraction procedures; complex matrices; non-conventional matrices; clinical and biological applications

1. Introduction

Nowadays, the possibility of having both sampling and sample clean-up procedures available, but also rugged and reproducible instrumental configurations in order to obtain clinical/biological data that can be compared, is increasingly important. In the field of Analytical Chemistry, both of these

points are essential. In particular, the first is the step that requires the greatest amount of time, while the second is the step that mainly can (or could) limit the sensitivity of the entire procedure [1,2].

With regard to sampling and extraction procedures, enormous advantages can be obtained by applying some general rules such as: reduction of sample handling in order to reduce the sources of analyte loss, applying procedures that allow obtaining high enrichment factors values, and using preparative methods as selective as possible with respect to the analyte/s of interest.

As far as the instrumental configurations are concerned, we have witnessed an increase in the complexity of the instrumental configurations gradually to more and more hyphenated, and an increase in the analytical performances of the instruments themselves (up to an instrumental sensitivity of the order of fmol) [3].

In addition, both factors taken together contribute to the selectivity of the method and, above all, to the improvement of the signal-to-noise ratio (S/N, related to the whole procedure sensitivity).

Another fundamental point that sees Analytical Chemistry in the foreground are the type of matrices on which quantitative analysis is necessary. In fact, recently, the analyses in the biological field have increasingly focused on unconventional matrices, the sampling of which appears to be as little invasive as possible, in order to be able to reduce any inconvenience in patients, but also to optimize the procedures during clinical studies [4,5].

In this abovementioned context, in addition to the classic plasma (or serum) matrices, biopsies, whole blood, and urine, we have seen an increase in the works that also consider salivary, keratin and sweat matrices. Obviously, all these studies have shown that there is a correlation between the blood levels and those found in the new matrix, in order to be able to correlate and compare the results in a robust way and reducing any bias problems.

Currently, there is also a growing trend in combining the "classic" conventional techniques with "micro" formats in order to respond to the problem of the low quantity of sample that we often find having to process, as well as a growing combination of micro-extraction techniques. This phenomenon often leads to a synergistic effect of the advantages of the single procedures, obtaining a combined technique that shows greater analytical performances [6], as also recently reviewed [7], as clearly reported in Figure 1.

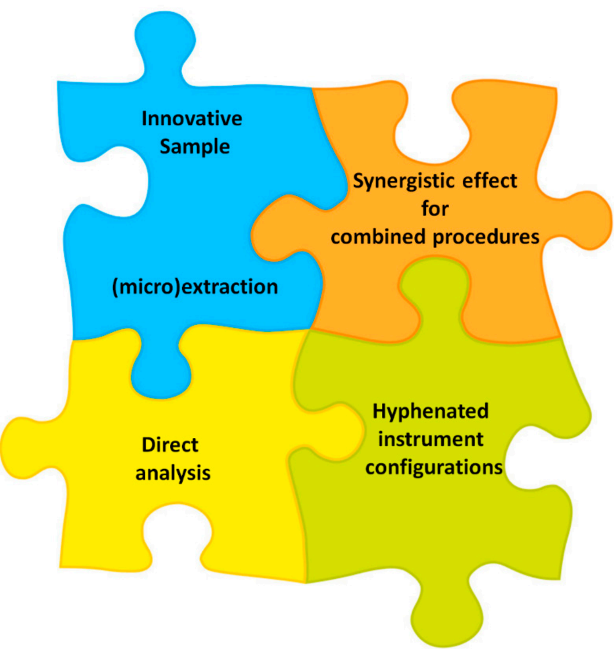

Figure 1. Connections between the various elements that, through their strengthening/improvement, will be able to face the challenges of the future in the bioanalytical field.

This review aims to report the most recent advances reported in literature in the recent years, and related to the elements just described, paying particular attention to all those procedures and instrumental configurations applied in the field of Bioanalytical Chemistry trying to highlight advantages and disadvantages, as well as the possible scale-up at the clinical/industrial level.

2. Pre-Treatment Procedures

When analyzing complex biological samples such as whole blood, plasma, urine, and biopsies, the sample preparation step is critical as it deals with complex matrices with moderate to high protein levels [8]. Another element in the case of biological matrices is represented not only by *inter-personal* variability, but also by *intra-personal* variability. Traditional procedures often require a lot of time and several steps in order to get a thorough cleaning before analysis [9,10]. To address these shortcomings, many innovations in extraction methods have been introduced in recent years [1].

Among the various biological matrices, urine is one of the most commonly used biological matrices (readily available [11] and *non-invasive* sampling), although it presents problems related in the direct analysis of this matrix. First of all, often significant quantities of the analytes (both of endogenous and exogenous origin) can be metabolized into different products or be converted by sulfonation or glucuronidation reactions into more soluble metabolites. Secondly, the urine shows a high content of dissolved inorganic salts, in addition to varying from lot to lot, the variable detection time window (which depends on the analyte) and the concentrations are strictly related to the type of diet followed by the subject. Another element that underlines the presence of intra- and inter-personal variability is linked to the fact that for urine pH values between 5 and 8 are reported with ionic strength, varying according to the level of hydration and the diet of the subject [12]. The complexity of the urine leads to difficulties for the direct analysis of this matrix, in addition to limiting sensitivity and selectivity.

The most used approach is certainly the one called "dilute and shot", where the sample is diluted in order to standardize and limit the matrix effect to obtain a better S/N ratio, in addition to decreasing the signal linked to the matrix and to the interferers. However, this procedure does not eliminate the interferences that are present in the instrumental analysis, which is therefore still partially influenced by ion suppression (or enhancement) if a hyphenated mass spectrometry (MS) technique is used.

In the extraction procedures applied to address these issues, various materials, coatings, configurations, and approaches have been developed to improve the sensitivity, selectivity, and extraction efficiency of the methods (in techniques such as solid phase extraction (SPE), solid phase microextraction (SPME), fabric phase sorptive extraction (FPSE)) towards analytes present, also at the level of traces and ultra-traces. These include the development of selective extraction phases obtained by molecular imprint polymers (MIP) to be used in not only the SPE configuration [13] or SPME, but also magnetic nanomaterials for bulk extractions [14], as also recently reviewed by Sajid [15]. The use of ionic liquids as coating materials that allow modulating the surface chemistry of the device [16] has also been recently investigated.

However, a fundamental element in recent years has been the development of new configurations of these techniques. In particular, the thin film SPME (TF-SPME) [17], which allows increasing the volume of the extracting phase in contact with the sample, as well as the stir-bar sorptive extraction (SBSE), and the *in-tube* SPME. These configurations allow increasing what is the highest surface/volume ratio, providing greater sensitivity and faster sampling times due to an increase in the extraction mass transfer process [18,19].

As Hashemi and coworkers reviewed in depth [20], SPE and SPME certainly represent the best known purification and pre-treatment techniques, even if both have in the past presented problems related to limited physical and chemical stability at low or high pH values as well as low capacity and limited reusability associated with conventional adsorbents. In addition to these complications, the clogging problem of the device often has to be faced, with a consequent reduction in extraction efficiency.

Worthy of note, however, is the FPSE technique, in which the main problems of conventional techniques (e.g., clogging) are easily overcome [1]. The FPSE successfully couples the advantages of

equilibrium-based extraction (SPME/SBSE) with the exhaustive extraction characteristic of SPE without the need for matrix pre-treatment [21]. In this technique, the adsorbent surface covalently bonded to the surface of the support has a high chemical, physical, and thermal stability, as well as offering a very high contact surface due to the open surface geometry, increasing the speed of the analyte absorption/desorption process. Furthermore, the various types of surface chemistry available for FPSE (as the bound phase is not inert) allow modulating the selectivity of the extraction process.

Together with urine, the blood matrix is undoubtedly the most used biological fluid in clinical analyses and pharmacokinetic studies. Most of the works in literature, however, consider only a part of it, either plasma or serum, leading to a loss of analytical information when a quantitative analysis is performed. The analysis of these matrices provides information on metabolites, drugs, biomarkers and exogenous toxic substances and involves the processing of a large number of samples in which the analytes are often found at the trace level. Another problem concerns the stability of the analytes that can undergo degradation reactions by enzymes, as well as degradation processes linked to pH variations, storage conditions, or the addition of anticoagulants [22]. In this type of matrix, procedures such as liquid–liquid extraction (LLE), protein precipitation (PP), as well as SPE and SPME are generally applied. Note that, also, in this case, exhaustive extraction techniques (LLE, PP, and SPE) and equilibrium-based extraction techniques (SPME) have been included. Furthermore, it should be emphasized that LLE and PP are not sufficiently selective, leading to a final supernatant that could contain high concentrations of matrix interferences, such as non-precipitated proteins, salts and phospholipids that could lead to ion suppression phenomena (or enhancement) in the case of hyphenation with the MS.

It should be emphasized that between LLE and PP, the first certainly allows to obtain a "*cleaner*" supernatant as it uses organic solvents (and therefore does not follow the rules of Green Analytical Chemistry (GAC)) that are not miscible with the sample and therefore, less subject to the presence of macromolecules and salts.

Up to now, techniques applied exclusively to plasma or serum have been reported, leading to an inevitable loss of analytical information. To address this problem, not only have techniques been developed such as the FPSE which allows the analysis of whole blood [23–26], but also the Dry Blood Spot (DBS) [27,28], and the Biofluids sampler (BFS) [21]. These devices allow the analysis of the "whole blood" matrix as it is, with small sample volumes (less than 20 μL), by means of non-invasive sampling (capillary blood).

A notable advantage of DBS and BFS is represented by the fact that the sample is directly deposited on support and stored at room temperature, therefore, it does not require any manipulation, as well as simplifying the procedures, and avoiding loss of analytical information linked to pre-production processes/treatment.

Although these devices show such advantages, they do not allow to clean the sample in depth (unless specific surface chemistry is selected), and generally require highly sensitive instrumentation due to the low volume of loaded sample, the potential degradation of some analytes due to storage conditions, and variable blood diffusion depending on the type of support. It should also be noted that the performances also depend on the hematocrit level, as the viscosity of the blood depends on it [29].

In the analysis of matrices such as plasma and serum, SPE, thanks to a wide range of adsorbent phases, is often the most used technique. However, this technique shows clogging problems, as well as not being able to be applied in the case of small samples (about 10–50 μL). In this context, it is possible to include another extremely useful technique, the micro-extraction on packed sorbent (MEPS). This technique represents a sort of miniaturization of the SPE, with the advantage of being able to use the same cartridge several times, as reported in Figure 2.

Furthermore, in the analysis of biological samples, the SPME technique shows limits linked to swelling phenomena of the membranes in contact with the organic solvents typical of LC analyses, limiting their applications. In addition to the matrices presented so far, in the bioanalytical field, it is often possible to run into unconventional matrices (e.g., saliva) [4], but also invasive samples such as

tissues and biopsies. The procedures applied to analyze tissues can be evaluated based on the sampling process, based on how the sample is processed and the time elapsed from sampling to analysis.

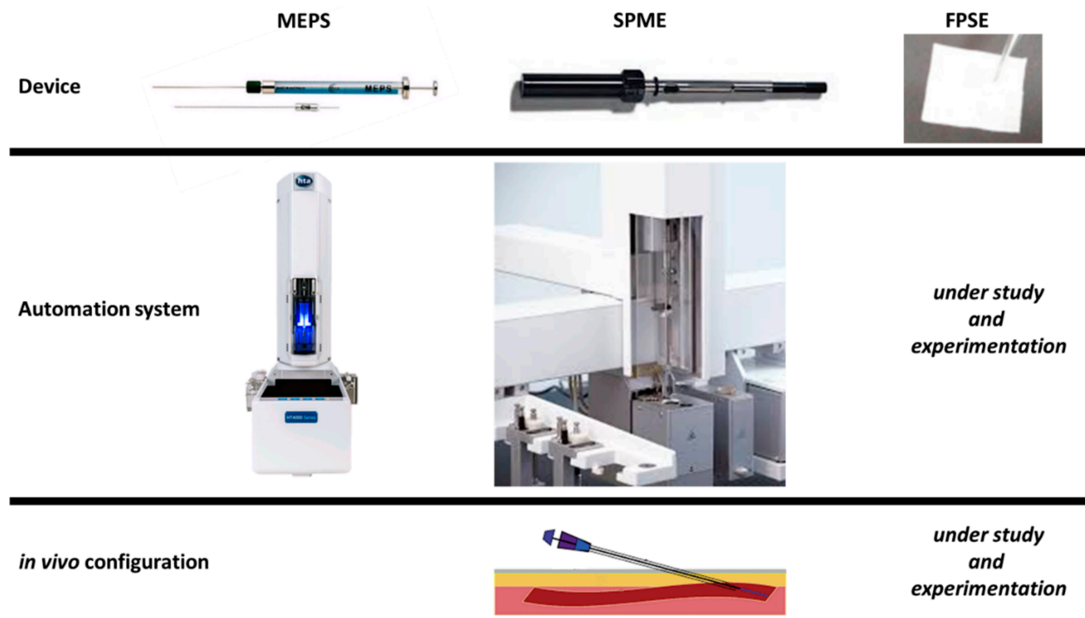

Figure 2. Current developmental stages and future perspectives of MEPS (micro-extraction on packed sorbent), SPME (solid phase microextraction), and FPSE (fabric phase sorptive extraction).

In general, in the case of such matrices, strategies such as lethal or non-lethal (ex vivo) removal, direct measurement with the aid of biosensors (or by probes or membranes) can be applied.

It should be noted that in the field of bioanalytics (especially in the chemical-clinical field), all the procedures implemented on the samples must be able to be controlled. This is to provide reproducible and robust results, and to avoid that in the chain of custody (as well as in the internal procedures of the laboratory), elements are introduced that could generate "artifacts" or methodological errors.

Despite the progress made in the field of pre-treatment procedures, there is still a lot of space to improve the procedures, increase selectivity and sensitivity, and reduce possible error funds. This is all with an eye towards the development of green procedures and the new tool to evaluate the greenness (GAPI—green analytical procedure index) [30], easily scalable, and applicable to all the different instrumental configurations. Specifically, not merely to the most modern and performing ones which require high purchase/maintenance/operating costs as well as than trained personnel, but also the most well-known, robust and widespread ones.

Table 1 shows the most important developments on extraction procedures and instrument configurations reported in the literature in the last years that meet the needs of selectivity, sensitivity and extraction efficiency of several analytes from complex biological matrices.

Table 1. Most important developments on innovative extraction procedures and instrument configurations in terms of selectivity, sensitivity and extraction efficiency of various analytes from complex biological matrices.

Matrix	Analyte/S	Extraction Configuration	Phase System	Instrument Configuration	Stationary Phase	Elution	Ref.
Saliva	NSAIDs	FPSE	PTHF	HPLC-PDA	C18	isocratic	[4]
Ovarian cancer tissue	NSAIDs	MASE-SPE	-	HPLC-UV	C18	isocratic	[6]
Human Whole blood	NSAIDs	BFS	-	HPLC-PDA	C18	isocratic	[21]
	Parabens	FPSE	CW20M	HPLC-PDA	C18	isocratic	[26]
	IBD drugs	FPSE	CW20M	HPLC-PDA	C18	gradient	[23]
	Aromatase inhibitors	FPSE	PEG-PPG-PEG	HPLC-PDA	C18	gradient	[24]
	UV filters	FPSE	CW20M	HPLC-PDA	C18	isocratic	[25]
	Antidepressants	SPME	PAN-C18	HPLC-MS/MS and DART-MS/MS	C18	gradient	[31]
	β-blockers	Ionic liquid-DLLME	-	HPLC-DAD	C18	isocratic	[32]
	Prostaglandin	DBS-MEPS	C18	UHPLC-ESI-MS/MS	C18	gradient	[33]
Rat whole blood	Antidepressants	In vivo SPME	PPY	HPLC-MS/MS	C18	gradient	[34]
Dog whole blood	Antidepressants	In vivo SPME	PEG-C18	HPLC-MS/MS	C18	gradient	[35]
Human Plasma	Parabens	FPSE	CW20M	HPLC-PDA	C18	isocratic	[26]
	IBD drugs	FPSE	CW20M	HPLC-PDA	C18	gradient	[23]
	Aromatase inhibitors	FPSE	PEG-PPG-PEG	HPLC-PDA	C18	gradient	[24]
	UV filters	FPSE	CW20M	HPLC-PDA	C18	isocratic	[25]
	Antidepressants	SPME	PAN/PS/DVB	HPLC-MS/MS	PFP	gradient	[36]

Table 1. *Cont.*

Matrix	Analyte/S	Extraction Configuration	Phase System	Instrument Configuration	Stationary Phase	Elution	Ref.
	Antidepressants	SPME	PTP (polythiophene)	HPLC-UV	C18 RP-Select B	isocratic	[37]
	Benzodiazepines	SPE-DLLME	-	HPLC-UV	C18	Isocratic	[38]
	β-blockers	MIP-SPE	-	HPLC-UV	C18	isocratic	[39]
	β-blockers	Magnetic SPE	Graphene nanocomposite	HPLC-UV	C18	isocratic	[40]
	β-blockers	SPMMTE	Iron nanocomposite adsorbent	UPLC-Q-TOF-MS	PFP	isocratic	[41]
	Endocannabinoids	Bio-SPME	HLB	nano-ESI-MS/MS	-	-	[42]
Human Serum	Antidepressants	SPME	C16 amide	HPLC-MS/MS	C18	gradient	[43]
	Vitamin D and analogues	*On line* SPE	C18	2D-UPLC-ESI-MS/MS	PFP-C18	gradient	[44]
Human Urine	β-blockers	SBSE	polymeric	HPLC-UV	C18	isocratic	[45]
	Parabens	FPSE	CW20M	HPLC-PDA	C18	isocratic	[26]
	Parabens	MIP-SPE	CW20M	HPLC-PDA	C18	isocratic	[13]
	IBD drugs	FPSE	CW20M	HPLC-PDA	C18	gradient	[23]
	Aromatase inhibitors	FPSE	PEG-PPG-PEG	HPLC-PDA	C18	gradient	[24]
	UV filters	FPSE	CW20M	HPLC-PDA	C18	isocratic	[25]
	Anabolic steroids	MIP-SPME	-	GC-MS	HP-5MS	temperature gradient	[46]
	Illicit drugs	Ionic liquid-SPME	-	GC-MS	DB-1MS	temperature gradient	[16]
	Illicit drugs	SPME	C18	DART MS/MS Coated blade spray-MS/MS DART-MS/MS	- - -	- - -	[47] [48] [49]

Table 1. *Cont.*

Matrix	Analyte/S	Extraction Configuration	Phase System	Instrument Configuration	Stationary Phase	Elution	Ref.
	Metabolic profile	SPME	PA	GC-MS	HP-5	temperature gradient	[50]
	Metabolic profile	SPME	DVB/CAR/PDMS	GC x GC-TOF-MS	HP-5 x DB-FFAP	temperature gradient	[51]
	Benzodiazepines	SPE-DLLME	-	HPLC-UV	C18	isocratic	[38]
	Pesticides and metabolites	QuEChERS	-	UHPLC–HRMS	Hypersil Gold aQ	gradient	[52]
	Veterinary drugs	QuEChERS-SPE	-	UHPLC–HRMS	Hypersil Gold aQ	gradient	[53]
	β-blockers	MIP-SPE	-	HPLC-UV	C18	isocratic	[39]
	β-blockers	MIP-SPE	-	HPLC-UV	C18	isocratic	[54]
	β-blockers	*On line* MEPS	-	HPLC-FLD	C18	gradient	[55]

HPLC-PDA: high performance liquid chromatography-photodiode array detector; HPLC-FLD: high performance liquid chromatography-fluorescence detector; GC-MS: gas-chromatography-mass spectrometry; GC x GC-TOF-MS: bi-dimensional gas chromatography-time of flight-mass spectrometry; UHPLC-HRMS: ultra-high pressure liquid chromatography-high-resolution mass spectrometry; DART-MS/MS: direct analysis in real-time-tandem mass spectrometry; 2D-UPLC-ESI-MS/MS: bi-dimensional ultra-performance liquid chromatography-electrospray ionization-tandem mass spectrometry; HPLC-MS/MS: high performance liquid chromatography-tandem mass spectrometry; UPLC-Q-TOF-MS: ultra-performance liquid chromatography-quadrupole-time of flight mass spectrometry; FPSE: fabric phase sorptive extraction; SPME: solid phase micro extraction; SPE-DLLME: solid phase extraction-dispersive liquid-liquid micro extraction; MIP-SPME: molecular imprinted polymer-solid phase micro extraction; QuEChERS-SPE: Quick, Easy, Cheap, Effective, Rugged and Safe; MEPS: micro extraction by packed sorbent; SPMMTE: solid-phase micro membrane tip extraction; SBSE: stir bar sorptive extraction; MASE-SPE: microwave assisted solvent extraction-micro solid phase extraction; BFS: biofluids sampler; Bio-SPME: biocompatible solid phase micro extraction.

3. Instrument Configurations

As previously reported for the extraction techniques, also from an instrumental point of view, massive progress has been made in terms of sensitivity and selectivity for quantitative analysis. Currently, several LC-MS hyphenated techniques are able to quantify analytes present at the ultra-trace level (fmol order). It should be emphasized that these highly performing configurations, often not within everyone's reach, allow not only obtaining an accurate (precise and true) quantitative analysis, but also allow exploring of previously unattainable concentration levels. Often, however, this instrumentation suffers from problems of response linearity over wide ranges of concentration, especially for analyses conducted in complex biological matrices, and above all, when the matrix components can act differently depending on the analyte concentration level.

The general principle of manipulating the sample as little as possible in order to avoid loss of analyte has also led to the development not only of complex chromatographic configurations like ultra-high performance liquid chromatography (UHPLC) or two-dimensional chromatography (2D-LC), but also to the development of new interfaces used when MS is coupled with separation methods. In particular, in the latter case, very promising (although still in the early stages of its development) perspectives were shown for the desorption electrospray ionization (DESI) [56,57], the extractive electrospray ionization (EESI) [58,59], the laser ablation electrospray ionization (LAEI) [60,61], and the solid-substrate electrospray ionization mass spectrometry (SESI-MS). The latter are an integral part of ambient ionization mass spectrometry (AIMS) techniques that generally require a minimum pre-treatment of the sample [62–64], as well as showing advantages in terms of high productivity as they speed up the analyses in MS. These approaches were performed by applying different substrates as ESI emitter, from metal [65,66], wood [67–69], paper [70,71], polyester [72,73], to polyethylene [74], graphene [75], and carbon fiber [76,77], to improve the sensibility and selectivity. Considering merely the chromatographic section, UHPLC and 2D-LC are certainly the most recent and promising configurations.

In this context, UHPLC-MS has certainly seen the greatest developments (almost at an exponential level) for applications both in the field of research, but above all, in routine analysis. This increase, largely linked to the reduction of analysis times (and consequently to lower consumption), led to a reduction in costs as well. This was made possible by an increase in MS performances and above all, in the signal acquisition speed (both in full scan and in MS/MS mode as well as the possibility of inverting the polarity of the ionization) which allowed to have residence times of 1 msec and polarity switching of 30 msec or less [78]. This coupling allows increasing the sensitivity (as well as the precision and trueness) as phenomena of increased resolving power, reduction of ion suppression and improvement of peak symmetry are observed [79]. From the point of view of environmental impact, a positive consequence of this coupling is represented by a reduction in the volume of chemicals (e.g., mobile phases) and waste, and it is more respectful of the environment [78].

All the more reason, these positive elements are further strengthened by the use of green sample pre-treatment procedures [80,81], as reported in the previous paragraph, or the development of fully automated methods, including compound extraction, separation and detection.

After seeing the progress in terms of performance related to one-dimensional chromatography (1D-LC) but at very high pressure, we now see other developments in terms of instrument configurations that will surely see an increase in applications in the near future. We mainly refer to two-dimensional chromatography techniques (2D-LC), which have allowed generating much higher peak capacities than 1D-LC [82–84]. It should be pointed out that the greatest advantages are mainly linked to the possibility of coupling two equal or different separation/elution conditions in the two dimensions (obtaining an orthogonal configuration of the chromatographic section) in order to increase separation and sensitivity. When working in 2D-LC, it is very important to understand that the use of two different separation mechanisms implies that the separation on the second dimension is the critical point. Often, the fractions deriving from the first dimension are in unsuitable (or not optimal) solvents for the second dimension, leading, rather than to an improvement, to a worsening of the chromatographic

performances. The purpose of the second dimension is to increase the sensitivity through an increase in the resolving power [84,85], as reported in Figure 3.

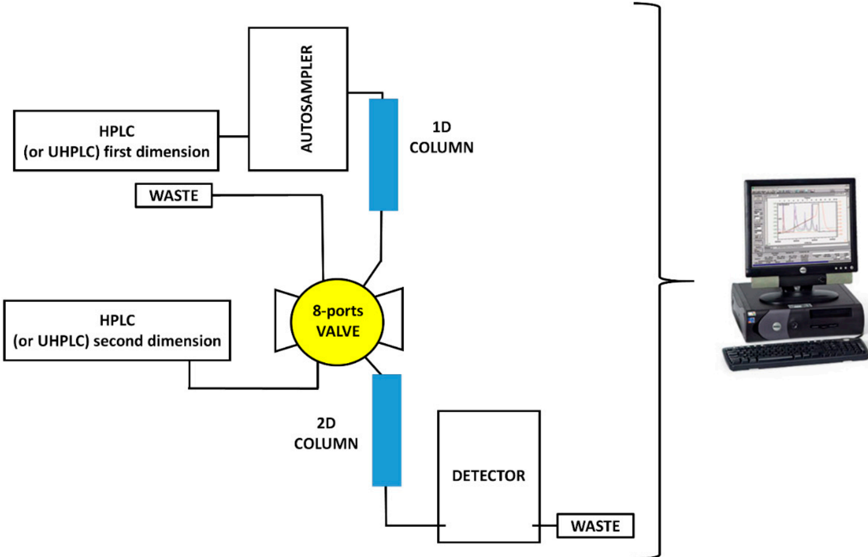

Figure 3. Typical 2D-HPLC (two-dimensional chromatography) (or UHPLC) instrument configuration.

The 2D-LC technique has seen a strong increase (and is a valid option), especially in those cases in which the number of components exceeds generally 37% of the peak capacity of the method and in which the peak resolution is statistically reduced [86]. To do this, the elution rate of the second dimension must be the maximum usable. For this, the use of 2D configurations with UPLC techniques are the most suitable. 2D configurations can be implemented either fully (LCxLC) or with heart-cutting (LC-LC). In the first configuration, the eluate of the first dimension is sent entirely to the second dimension, while in the second (LC-LC), one or more segments of the chromatogram of the first dimension are sent to the second dimension. Obviously, the LCxLC mode sees the times coupled while in the LC-LC—they are "out of phase" and there are no time constraints regarding the separation on the second dimension [87]. Several innovative instrument configurations that show these characteristics were reported in Table 1.

The advancement of extraction protocols and the development of advanced instrumental configurations has allowed the application of analytical methods in clinical practice. The development of sensitive methods, particularly in the analysis of biological fluids (plasma, urine, whole blood, saliva, etc.) has become the focus in several fields of research. There has been an impressive emergence mostly of mass spectrometry technologies applied toward the biomedical approaches. Mass spectrometry represents, nowadays, the primary method in different studies, and some examples could be the drug development process [88], the newborn screening [89–91], the doping control [92] and the detection of biomarkers for various diseases (proteomic, lipidomic and metabolomic) [93–97]. From these efforts come the promise of better diagnostics/prognostics, mostly for that disease that does not have a valid therapeutic approach, such as cancer, neurodegenerative disorders and many others.

Another very important element that we are starting to consider more and more nowadays, is the green approach, right from the development phases of a method, obviously always considering the performances of the procedure as basic. Regarding this, Ballester-Caudet [98] and Płotka-Wasylka [30] have recently reported very interesting applications on GAPI that allow an evaluation in this sense.

4. Conclusions

As can be seen from this review, many advantages linked to the use of innovative (micro)extraction techniques, in addition to the use of specific instrumental configurations, include factors such as:

- ✓ better selectivity towards molecules with different chemical-physical properties,
- ✓ reduction of matrix effects (in the case of hyphenated techniques),
- ✓ greater flexibility in terms of sample volume required,
- ✓ ease of use and suitability of automation,
- ✓ simplification of the sample preparation procedure (up to complete non-pre-treatment of the same),
- ✓ feasibility for the determination of free and total concentrations
- ✓ possible "synergistic effect" related to the techniques combination.

It is also observed how this field is constantly evolving and that new and more performing devices are being developed to meet the growing (and often different) needs that every analytical chemist has to face. We would like to highlight that what is reported in this review actually represents only the "tip of the iceberg" and that no reference has been made to the applications of chemometric models in the bioanalytical field. Just a small parenthesis to highlight how even these statistical/mathematical approaches regarding the processing of raw data deriving from instrumental analyses have seen growing interest in recent years [99,100] and an expansion of the application fields.

In the future, biocompatible protocols and devices will be increasingly required for in vivo monitoring of analytes, as well as devices that allow in situ and *non-invasive* sampling in order to implement current knowledge with data obtained directly on the subject, without manipulation (and therefore possible contamination) of the sample. Furthermore, given the wide range of biological fluids (and tissues), the challenge is open in the development of universal techniques that can be applied on multiple types of biological matrices.

Likewise, recently, the Scientific Community started talking about the possibility of *real-time monitoring* directly in the operating room or the so-called *point of care*, directly obtaining the quantitative analysis in a very short time in order to offer the patient accurate monitoring and with a significant reduction of waiting times. All these elements inevitably lead to the need for a continuous effort on the part of Analytical Chemists to develop new procedures, devices, and instrumental configurations capable of responding to these needs.

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