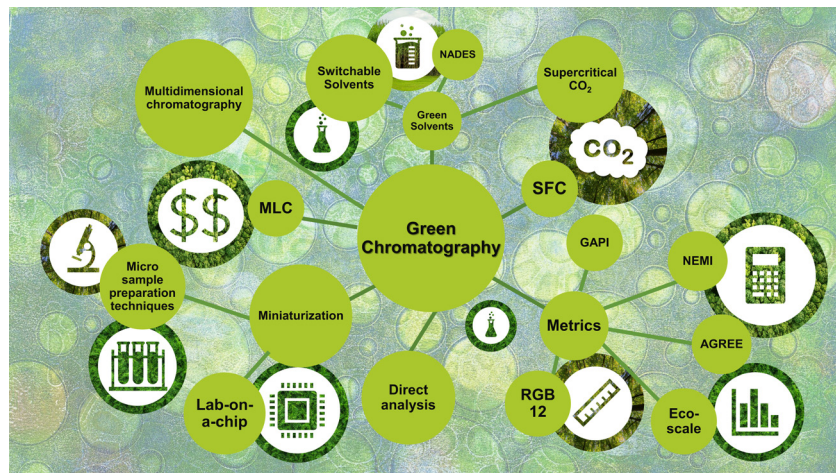


REVIEW

Recent Developments in Green Chromatography

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In recent decades, green analytical chemistry has received more attention due to the growing concern over environmental conservation and the use of non-renewable resources. Among the analytical techniques, liquid chromatography is the most widely used in quality control analysis of food, drugs, and clinical analysis among others, but it is also the technique that uses the largest amount of hazardous organic solvents and generates large volumes of waste. Therefore, strategies such as the

miniaturization of chromatographic systems, the use of online sample preparation systems, and the replacement of hazardous organic solvents by green solvents have been applied to develop greener chromatographic methods. In this paper, strategies for greening methods and recent developments in green chromatography are presented. In addition, metrics for the proper evaluation of these methods are discussed.

Keywords: chromatography, green analytical chemistry, greenness assessment, miniaturization, sustainability

INTRODUCTION

The concept of green chemistry emerged in the early 1990s to mitigate the hazards posed by chemicals to the environment and human health.¹ To this end, Anastas and Warner proposed the 12 principles of green chemistry as guidelines for improving chemical systems, processes, materials, and products, and applied them primarily to industrial chemical processes.² Later, the IUPAC (International Union of Pure and Applied Chemistry) defined green chemistry as “*The invention, design, and application of chemical products and processes to reduce or to eliminate the use and generation of hazardous substances*”.³ These principles were elaborated for synthetic chemists and at the industrial level at the first moment due to their large scale, large amount of waste produced, and therefore high impact on the environment, when compared to analytical chemistry.

However, the environmental impact generated by analytical laboratories nowadays is high considering that thousands of analyses are performed in a single day.⁴ Thus, the application of green chemistry

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principles in analytical chemistry laboratories is fundamental to reducing the environmental impact and guarantee safety to the analysts. The application of green chemistry principles in analytical chemistry can be defined as Green Analytical Chemistry (GAC), seeking the development of new methods and techniques capable of reducing the use and generation of hazardous substances in all stages of chemical analysis. However, only four of the twelve principles of green chemistry could be applied directly to GAC once they were planned to attain the needs of industrial and synthetic chemistry.⁵ In this context, in 2013, Galuska *et al.* adapt the 12 principles of green chemistry and proposed the 12 principles of the GAC which are: (1) Direct analysis techniques should be employed to avoid sample treatment; (2) The number and size of the sample should be minimal; (3) Perform in-situ measurements; (4) Integrate analytical procedures and operations to save energy and reduce reagent consumption; (5) Select automated and miniaturized methods; (6) Avoid derivatizations; (7) Avoid the generation of large volumes of waste and dispose of it properly; (8) Use multi-analyte or multi-parameter methods; (9) Energy consumption must be minimized; (10) Use reagents from renewable sources; (11) Toxic reagents must be eliminated or replaced; (12) The security of the analyst should be increased.⁶

According to the GAC principles, the development of analytical methods should cause a minimum impact on the environment and should be safe for the analyst. However, the GAC principles implementations may lead to significant challenges regarding analytical method validation. For example, the reduction of sample size or the number of samples in the sampling process may cause a loss of the method representativity, precision, trueness, selectivity, and sensibility. As a consequence, the main challenge to future applications of the GAC is to reach a compromise between the validation parameters of analytical methods and the method's sustainability.

Liquid chromatography (LC) is a powerful and widespread separation technique that has been routinely used for the determination of organic compounds in complex mixtures at very low concentrations. However, substantial sample cleanup is needed for LC procedures, and large amounts of organic solvents and reagents are used during analysis. In light of this, these features of LC methods can be enhanced by implementing strategies based on the GAC principles into practice.⁷ Thus, the application of concepts of GAC in chromatographic techniques can be called green chromatography.

GREEN CHROMATOGRAPHY

Green chromatography generally aims to eliminate or reduce toxic solvent consumption, reduce analysis time, ensure analyst safety, and reduce waste generation. Several strategies can be employed to meet these objectives such as using miniaturized chromatographic techniques, performing on-site measurements, using green solvents as mobile phases, integrating multiple steps of the analytical procedure into a single step using hyphenated techniques, using automated methods, and when possible, using direct analysis.^{7,8,9} As it is known, high-performance liquid chromatography (HPLC) uses large amounts of organic solvents such as acetonitrile and methanol, producing large volumes of waste. From the GAC's perspective, the simplest approach in this circumstance would be to minimize solvent consumption or replace it with less harmful or biodegradable solvents. Glycerol, for example, is derived from renewable sources and is a low-cost green solvent (biodiesel byproduct) that is non-volatile and stable under normal storage circumstances. And recently, mobile phases based on glycerol and an aqueous buffer solution were reported in the literature for detecting ascorbic acid and glutathione in pharmaceutical tablets.^{10,11} On the other side, gas chromatography (GC) does not use solvents in the separation process. Hereby, the use of new technologies for temperature control and modifications to sample preparation must be carried out for the development of green methods. Other chromatographic modes that can minimize the consumption of hazardous organic solvents in the mobile phase include supercritical fluid chromatography (SFC) and enhanced fluidity liquid chromatography (EFLC). SFC is comparable to HPLC, except that the mobile phase is mostly composed of fluid at or near its critical temperature and/or pressure. These fluids are more gas-like than normal liquids, with increased solute diffusivity and decreased viscosity. As a type of normal phase chromatography, SFC uses non-polar fluids like carbon dioxide.¹² Polar organic solvents are added

to the fluid to increase the mobile phase polarity to 30 to 40%, and when the percentage of organic solvent is higher, it is referred to as EFLC.¹³

In terms of sample preparation, for example, microextraction techniques using less hazardous solvents¹⁴ and the use of innovative sorbent materials with lower cytotoxicity and biocompatible supports are the main topics of recent studies in the development of green chromatographic procedures.^{15,16} These current studies also attempt to create reusable instrumentation that ensures analytical performance equivalent to or better than the systems already in use.¹⁵

Green chromatography techniques are frequently cost-effective because they support protocols that require minimal or reduced solvent or reagent requirements. Therefore, the chromatographic methods have the potential to be greener in all analysis steps, from sample preparation to the separation and the final determination. Moreover, when developing a method in agreement with GAC principles, it is necessary to determine whether the strategies employed in its development have made it greener. Thus, the evaluation of method greenness is performed by qualitative, semiquantitative, and quantitative metrics, which allows us to compare different methods and choose the greenest option. In this regard, this paper discusses several ways for making GC and HPLC greener, such as direct analysis and miniaturization of sample preparation and chromatographic system, as well as the available metrics for method greenness assessment.

Tools for method greenness assessment

The first developed metric for green chemistry was the National Environmental Method Index (NEMI),¹⁷ which judges the method greenness through a pictogram divided into four quadrants PBT (persistent, bioaccumulate, and toxic), Hazardous, Corrosive, and Waste.

The respective quadrant is filled with green color when the method meets the established criteria and the method with the most filled quadrants is the greenest one. Despite the simple representation and easy understanding, NEMI provides qualitative results and does not consider the reagent number used, the occupational hazards, and the equipment energy consumption. Therefore, according to GAC principles, this metric does not include all the parameters necessary to assess if a method is eco-friendly.

A semiquantitative evaluation can be done employing the Analytical Eco-scale (Eco-Scale)¹⁸ metric, which is a score classification system. An ideal green method starts with 100 points and penalties are applied to this score for each parameter such as reagent amount, hazardous, energy consumption, and waste generation that diverges from GAC ideality.

The Green Analytical Procedure Index (GAPI)¹⁹ is a tool that uses a pictogram to classify the method's greenness in each step of the analytical procedure, using a color scale with three levels of evaluation. In GAPI a specific symbol with five pentagrams is used to assess the levels of environmental impact involved in each step of the methodology, with the green color meaning low impact, yellow representing intermediate impact, and red as high impact. Each field reflects a different aspect of the analytical procedure described and it is represented by a number referring to the GAPI parameter description (Figure 1).

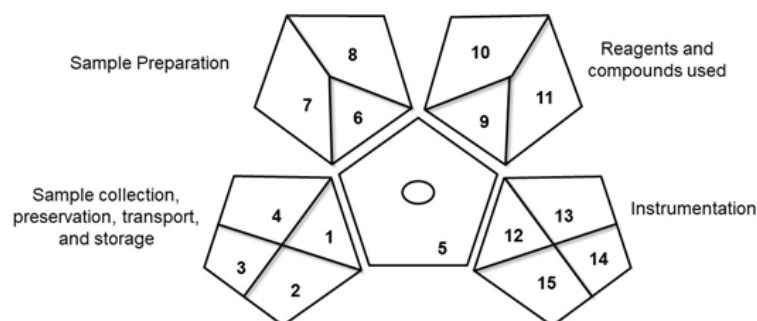


Figure 1. Representation of the GAPI pictogram. [Reprinted with permission by CCC Rights Service, from Ref. 19: Plotka-Wasyłka, J. A New Tool for the Evaluation of the Analytical Procedure: Green Analytical Procedure Index. *Talanta* **2018**, *181*, 204–209. DOI: 10.1016/j.talanta.2018.01.013. Copyright© (March, 2023), Elsevier].

The Eco-scale and GAPI metrics comprehended parameters that are not included by NEMI as several reagents employed and energy consumption. However, the GAPI metric provides a more complete evaluation than Eco-scale by detailing the analytical procedure from sampling, transportation, and sample preparation until the final determination of analytes. Despite the details provided by GAPI, the pictogram obtained is complex, and qualitative and does not assess the analytical method performance.¹⁹

A quantitative evaluation can be realized by Analytical Greenness Calculator (AGREE),²⁰ a comprehensive, flexible, and direct metric that provides an informative result and is easily interpretable. In this free software, there are twelve evaluation criteria based on the twelve principles of GAC, which are converted into scores ranging from 0 to 1. The final score result is the product of each principle's assessment results. The final assessment is presented as a clock shape pictogram (Figure 2), indicating the final score and the color in the clock center.²⁰

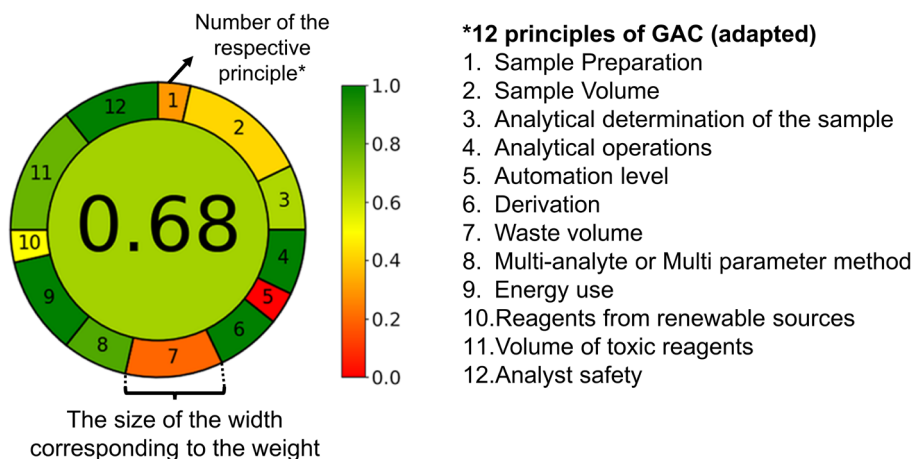


Figure 2. Representation of the AGREE pictogram. [Reprinted from Ref. 20: Pena-Pereira, F.; Wojnowski, W.; Tobiszewski, M. AGREE - Analytical GREENness Metric Approach and Software. *Anal. Chem.* **2020**, 92 (14), 10076–10082. DOI: 10.1021/acs.analchem.0c01887, an open access article published under a CC-BY License, which permits unrestricted use, distribution and reproduction in any medium.]

Of the metrics introduced, the AGREE metric is the one that best incorporates all GAC principles. However, it does not assess the number of reagents used in the method. Besides that, the introduced metrics do not consider the analytical method performance parameters, very important to the analytical validation step, and the practical viability of the method.

Due to the lack of a tool or metric capable of performing a comprehensive analytical method evaluation together with its green aspects, Nowak and Kóscielniak proposed an original model based on the RGB additive color model that allows a global method evaluation that can be performed in an Excel spreadsheet.²¹ The RGB additive model color commonly is used in electronic systems to represent and display colors and the name refers to the red, green, and blue colors, respectively. In the proposed RGB model the colors represent the primary attributes of analytical methods. The red color corresponds to the method performance assessed by analytical validation, the green color represents the GAC principles and the blue color refers to the productivity effectiveness which includes cost and time effectiveness, the complexity of methodology, trained staff, and equipment maintenance. To quantitatively assess whether the method conforms to the three main colors of the RGB model, a color score (CS) is calculated and ranges from 0 to 100%. If the evaluated method receives a CS higher than 66%, i.e., is rated as satisfactory, for all three colors the final color of the method will be white. This means that the method meets all the evaluated criteria, analytical performance, practical effectiveness, safety, and environmental friendliness. When the method

reaches $CS \geq 66.6\%$ for only two colors and results in color with $33.3 \leq CS \leq 66.6\%$, i.e., is classified in a tolerable value range, the final color of the method is a mixture of the colors that reached the satisfactory level. The colors resulting from the mixtures are yellow, (a combination of the color green and red), cyan (blue and green), and magenta (blue and red). If the CS of a method reaches the satisfactory level for only one of the colors, the final color of the method will be red, blue, or green. If the method has $33.3 \leq CS \leq 66.6\%$ for all three main colors the final color of the method will be gray (no color) and indicates that the use of the method is not recommended if a better method is available. However, if at least one CS is less than 33.3% the method gets the color black because at least one of the main attributes of the method is unacceptable.

Another parameter of the RGB model that can be used to evaluate the methods is the method brilliance (MB), which can be calculated by the geometric weighted average of the individual values achieved by the method. Thus, the method with the highest MB will be the one with the highest individual CS values. The RGB model was the first model proposed by the authors.²¹

Recently, Nowak *et al.* formulated the 12 principles of white analytical chemistry (WAC) to implement the principles of sustainable development (SD) in analytical chemistry due to the need to find an equilibrium between method greenness, its potential usefulness, and analytical performance. Just as sustainable development is based on three pillars (economic, social, and environmental), the 12 principles of the WAC were divided into three complementary areas (Figure 3). As in the RGB model, a white method contemplates the three colors and represents a balanced analytical methodology suitable for its intended use.²²

Red Analytical Efficiency	Green Safe and Eco-Friendly	Blue Economical and Practical Aspects
R1 – Scope of Application R2 – Limit of detection and quantification R3 – Precision R4 - Accuracy	G1 – Toxicity of reagents G2 – Number and amount of reagents G3 – Energy G4 – Direct Impacts	B1 – Cost-efficiency B2 – Time-efficiency B3 – Requirements B4 – Operational Simplicity

Figure 3. The 12 principles of white analytical chemistry are based on the RGB model. The 12 principles of green analytical chemistry were summarized into four main principles and complemented with four red principles and four blues principles representing analytical performance and practical/economic criteria, respectively. Adapted from Nowak *et al.*²²

The authors also proposed a new version of the RGB model and named it RGB 12. The new model is still based on free Excel spreadsheets and has been simplified for easy and quick evaluation of a method against each of the 12 proposed WAC principles. And thus assess the level of sustainability of the method, i.e., the whiteness of the method.²²

Strategies and current improvements to make chromatographic procedures more environmentally friendly

Green strategies applied to sample pre-treatment

Sample preparation is considered the step of the analysis with the greatest polluting potential, as it generally requires the use of organic solvents in large volumes. Sample preparation is also considered a crucial part of the analytical procedure based on quantitative determinations, identification, and chromatographic separations of a wide spectrum of analytes, especially in samples characterized by a matrix with complex composition.^{7,23} There are several ways to make sample preparation greener such as eliminating or reducing the number of organic solvents and reagents used in the analysis or the application of green solvents and recovery and reuse of solvents. The main advances related to sample preparation techniques applied to chromatographic methods are concentrated in the search for greener solvents such

as ionic liquids (IL), natural eutectic solvents (NADES), and supramolecular solvents (SUPRA), to replace the organic solvents used in microextraction. In this context, a class of green solvents called switchable solvents has been used for extraction purposes.²³ Switchable solvents are defined as solvents whose properties change abruptly in response to an external *stimulus*, such as a change in temperature or by purging or removing a gas. Usually, the term switchable solvents are related to tertiary and secondary amines that can switch their hydrophilicity as their structure changes in solutions due to pH changes.²⁴ For example, under normal conditions, switchable hydrophilic solvents are liquids that are so hydrophobic that they are immiscible with water and form a two-phase mixture. However, when CO₂ is added to the two-phase solvent/water mixture, the hydrophobic nature of the solvent significantly changes and becomes hydrophilic, which mixes with water to form a homogeneous mixture and increases extraction efficiency.²⁵ After the extraction process, an alkali treatment removes the CO₂, and the system reverts to a two-phase state, allowing the analytes to be easily removed and injected into the chromatograph.²⁶ Other switchable properties of these solvents include polarity, ionic strength, and surface activity.²⁴ In a recent study, Karayaka *et al.*²⁵ developed a method for the determination of alkylphenols and bisphenol A at trace levels using GC-MS after preconcentration with switchable liquid-liquid microextraction (SLLME) with N, N-dimethylbenzoamine as a switchable solvent. With the SLME-GC-MS method, the detection limits of the four analytes investigated remained in the range of 0.13-0.54 ng mL⁻¹, with an increase in detection power of 86- to 113-fold as compared to the detection limits reported by direct GC-MS. Recovery studies in tap water and wastewater were used to determine the method's accuracy and applicability in real samples, and the results ranged from 87 to 106%, showing that the method could be easily applied in routine water quality analysis. Furthermore, with this sample preparation, it was possible to reduce the consumption of solvents, and the generated residues were less toxic, making the method economical and compatible with the principles of the GAC, with a score of 88 on the Eco-scale. Other studies also presented greener chromatographic methods employing switchable solvents in microextraction techniques, as shown in Table II. Although the secondary and tertiary amines required for switchable solvent extraction are more expensive than ordinary organic solvents, the extractions are carried out on a micro-scale, and the extraction process is simplified, requiring only the addition/removal of CO₂ and a subsequent centrifugation step.

Table II. Recent applications of switchable solvents in sample preparation

Analytes	Matrix	Extraction Technique	Extraction Solvent/Sorbent	Analytical Technique	Mobile Phase	Benefits	Metric Employed
BFA ²⁵	Plastic bottles	LLE ^a	N,N-dimethylbenzamine	GC-MS	He	Waste with low toxicity Inexpensive and non-hazardous reagents	Eco-scale 88
Methamphetamine ²⁷	Urine	SHS - HLLME ^b	Dipropylamine	GC-MS	He	Quick extraction Reduced analysis time	NA ^g
Polybrominated diphenyl ethers ²⁸	Fish oil	VALLME ^c	different DES	GC-MS	He	Environmentally friendly and economical Low toxicity substances	NA ^g
Chlorobenzenes ²⁹	Water	HLLME	monoethanolamine/ 4-methoxyphenol ^f	GC-MS	He	Biodegradable solvents Fast and efficient extraction	NA ^g
Non-steroidal anti-inflammatory drugs ³⁰	Drinking Water	D- μ -SPE -HLLME ^d	Dispersive Solid - D- μ -SPE/Dipropylamine	HPLC-UV	ACN + 0.1% acetic acid	Reduced analysis time Environmentally safe	NA ^g
Tetracyclines ³¹	Urine	SP-LPME ^e	Fatty Acids ^f	HPLC-UV	Elution gradient ACN and MeOH (2:1) + 0.5% formic acid	Cheap reagents	Eco-scale 85

^aLiquid-liquid extraction; ^bHomogeneous liquid-liquid microextraction with switchable hydrophilicity solvent; ^cVortex-assisted liquid-liquid microextraction; ^eMembrane liquid-liquid microextraction; ^fDES; ^gNot applied.

In another study Wang *et al.* used for the first time NADES solvents as a matrix medium to replace DMSO, DMF, DMA, and water in a static headspace gas chromatography method for the determination of residue solvents in active pharmaceutical ingredients (API).³² The validated method showed an $R^2 \geq 0.999$ and provided high sensitivity with limits of detection (LOD) between 0.06 and 0.12 $\mu\text{g g}^{-1}$ for acetone, methanol, ethanol, isopropanol, n-butanol, acetonitrile, tetrahydrofuran, and 1,4-dioxane. Accuracy and precision showed acceptable results with recoveries of the tested solvents in the range of 94.3% to 105.4%, and relative standard deviations (RSD) ranged from 0.85 to 3.65 for intra-day precision and 1.51 to 4.53 for inter-day precision. The new approach was also used to successfully detect residual solvents in six APIs, sitagliptin, ramipril, imatinib mesylate, lisinopril, pramipexole dihydrochloride, and rivaroxaban.³² The replacement of these hazardous solvents with NADES provided a more eco-friendly and safer method from the GAC point of view, while not compromising the analytical method performance. However, no metrics were used to evaluate the greenness of the method. Sereshti *et al.* developed a novel micro QuEChERS/GC-MS method for the multi-class analysis of pesticide residues in cereal flour samples. The authors synthesized a nanofiber from a polymerizable eutectic solvent composed of poly (2-hydroxyethyl methacrylate):1tetradecanol and polyamide 6 and applied it for the first time as a sorbent in micro solid phase extraction. The proposed method reduced the amount of sample and the consumption of organic solvents (21%) needed for analysis while keeping the analytical performance similar to the methods referenced in the study. In addition, the proposed method was evaluated by two metrics, Complex GAPI, and Analytical Eco-Scale with a score of 72.³³ This method is a good example of how green strategies such as miniaturization, the use of green materials, and the reduction of solvent consumption can be combined to obtain a greener method without compromising analytical efficiency.

Miniaturization of instruments and reduction of the analytical scale of operations and increasing the efficiency of sample preparation with increased temperature and/or pressure or the use of microwaves and ultrasound techniques are also alternatives to make the process greener.^{34,35}

Methods with online sample preparation have also gained prominence in recent years due to reduced analysis time, manual operations, and sample quantity compared to offline sample preparation techniques.³⁶ The use of analytical methods that integrate the steps of sample preparation, separation, and detection reduces the number of reagents needed for the analysis, increases analyst safety, and minimizes the migration of solvents into the environment.³⁷ In addition, because online methods are automated, they ensure better accuracy and sensitivity, as well as reduce analysis time, meeting at least 4 of the 12 principles of the GAC.³⁸ However, as underlined by RGB 12 concepts, simply implementing these measures won't be enough to convince the industry and academia to follow GAC principles. The selected solutions must also be financially viable, primarily in terms of cost and time effectiveness and operational simplicity. In addition, the GAC principles and practices must be encouraged and regulated by specific legislations to further drive the adherence of industry and academia to GAC.

In this context, Jin *et al.* performed a study comparing the liquid-liquid extraction method and liquid chromatography coupled to mass spectrometry (LLE-LC-MS/MS) with the online extraction method with supercritical fluid combined with fluid chromatography supercritical coupled to mass spectrometry (SFE-SFC-MS/MS), for the determination of lipids in rat brain tissues.³⁹ The authors optimized the extraction by performing only 17 experiments using the Box-Behnken experimental design and the response surface methodology. As a result, the proposed SFE-SFC-MS/MS online method provided excellent performance compared to the LLE-LC-MS/MS offline method. The use of the design of experiments in extraction contemplated the reduction in the number of experiments and consequently saved time, energy, and reagents, while the online extraction system complied with the principles of GAC by integrating sample preparation and analysis, automation, and as consequence fewer manual steps were required, increasing operator safety and saving time and energy. All requirements met according to the GAC were proven by the AGREE algorithm by comparing the score of each method 0.75 and 0.49, for the online method SFE-SFC-MS/MS and the offline method LLE- LC-MS/MS, respectively.

Zhang *et al.* also developed an automated method combining online Solid Phase Extraction (SPE) and bidimensional liquid chromatography (LC-LC) for the simultaneous determination of vitamin A, D, and 4 vitamin E homologs in foodstuffs. In this method, the target analytes were released from their lipoprotein-coated state after saponification. Then, the saponified solution was injected directly into the system built by combining SPE (PLRP-S column) online and 2D-LC for pre-concentration, purification, separation, and quantification.⁴⁰ Vitamin A and vitamin E homologs were separated in the first column with pentafluoro phenyl (PFP) stationary phase and the separation of vitamin D was performed in the second column composed of polycyclic aromatic hydrocarbons (PAH). Evaluation of the results showed that this method not only fully met the requirements of analysis of vitamins A, D, and E in fortified foods or dietary supplements, but also had significant advantages over conventional liquid chromatography methods with ultraviolet detection (LC-UV) or LC-MS/MS in terms of method repeatability and recovery. Due to the simplification of sample preparation, the analysis efficiency has been significantly improved and the analytical cost has also been reduced. In addition, it is a green method since it avoids the use of pentane or other organic reagents. Therefore, the proposed method covered the six main trends of current analytical methods which are simplification, speed, cost and waste reduction, automation, and safety. However, no metric was used to verify the green aspects of the method.

Direct chromatographic analysis

In direct chromatographic analysis, the sample is introduced into the chromatograph without sample preparation to reduce the consumption of reagents and energy. However, methodologies of direct analysis involving complex matrices can damage the chromatographic columns due to the sample components that do not elute from the column. In general, the introduction of aqueous samples in GC capillary columns is not recommended as water and polar solvents can cause column bleed and can affect the performance of the column when samples are injected in the mode on-column resulting in a negative impact on the sensibility of the detector. An alternative to circumvent these limitations is the use of injectors with programmed temperature vaporization (PTV), the application of liners filled with sorbent material, or the installation of deactivated columns before the chromatographic columns. Temperature is one of the most important parameters that can be controlled in GC and its programming is widely used to improve the detection limit or peak symmetry, and results in a significantly reduced analysis time. This parameter can have an even greater positive environmental impact with the implementation of low thermal mass (LTM) technology. This technology utilizes an LTM column module combining a fused silica capillary column with heating and temperature sensing components wrapped around the capillary. Due to the small size of the system and its low heat capacity, the increase in capillary temperature requires smaller amounts of heat and therefore less energy compared to conventional GC ovens.⁴ The energy consumed using LTM technology is approximately 1% of the energy consumed by a conventional GC. In addition, it is possible to program temperatures with a rate of 1800 °C min⁻¹ heating and shorter cool-down times, resulting in reduced analysis times.⁴¹

Direct injection of aqueous samples can be performed in LC systems as long as the sample is filtered to prevent tube obstruction and damage to the chromatography column. As recommended by GAC, this approach involves minimal sample pre-treatment since most applications require only one dilution step with organic solvent and filtration before chromatographic analysis, ensuring less exposure of the analyst to these solvents. From this perspective, Dias *et al.* developed a fully validated ultra-high performance liquid chromatography-mass spectrometry (UHPLC-MS/MS) method for the simultaneous determination of 162 pesticides and 10 mycotoxins in wine samples with minimal sample pretreatment.⁴² Another example of direct chromatographic analysis is proposed by Restrepo-Vieira *et al.*, which developed and validated a UHPLC-MS/MS method with direct injection for the analysis of psychopharmaceuticals and illicit drugs in wastewater from Australia. The authors used 15 deuterated analytical standards to circumvent matrix effects and thereby reduced the analysis time, the number of consumables, and the production of residues when compared to methods using the SPE technique.⁴³ Nevertheless, the use of deuterated analytical

standards is not interesting from the economical point of view. According to GAC, these examples of direct chromatographic analysis provide simpler methods with less reagent consumption and less exposure of the analyst to hazardous organic solvents, as well as analytical performance comparable to methods that use pre-concentration and extraction techniques.

Replacement of toxic mobile phase solvents

The conventional mobile phase composition of Reversed Phase Liquid Chromatography (RPLC) consists of mixtures of acetonitrile or methanol in water. Both acetonitrile and methanol are toxic solvents and disposal of acetonitrile is expensive. Thus, these solvents must be replaced by greener alternatives such as water, acetone, and ethanol.^{44,45} Acetone has already been explored as a solvent to replace acetonitrile as a mobile phase in RPLC for the separation of peptides without any equipment modification.^{46,47} Recently, two studies on the solvation and retention properties of acetone have highlighted its potential to be employed as mobile in columns with typical octadecyl siloxane-bonded silica, octyl siloxane-bonded silica, and biphenyl siloxane-bonded silica phases.^{48,49} The only disadvantage of using acetone as a mobile phase in RPLC is the UV cut-off at 330 nm which makes it impractical to use UV-based detectors. However, for liquid chromatography coupled with evaporative light scattering detection, mass spectrometry, condensation nucleation light scattering detection, and charged aerosol detection, the use of acetone is a promising alternative. Ethanol was also explored as a mobile phase in RPLC applications. Dogan *et al.* have demonstrated that the analysis of drugs such as paracetamol and famotidine can be performed by green chromatography techniques, using a mobile phase based on ethanol-water, without losing any chromatographic performance and fulfilling all the requirements of the validation process.⁵⁰ Ethanol water-based mobile phase was also used in the determination of eight water-soluble vitamins in cosmetics.⁵¹ Organic solvents can also be replaced by supercritical fluids, and superheated water,³⁰ in this case, there is a change in the chromatographic technique. The analysis is performed on appropriate SFC equipment such as the ACQUITY UltraPerformance Convergence Chromatography (UPC²) (Waters), the 1260 Infinity II SFC/UHPLC Hybrid System (Agilent), and the Nexera UC (Shimadzu). CO₂ is the gas most used supercritical fluid due to its properties such as non-inflammability, low critical pressure (7,38 MPa), and temperature (31 °C).⁵² Besides that, CO₂ is an industrial subproduct of renewable resources. The supercritical CO₂ as a mobile phase in SFC exhibits the advantage of having solvent properties similar to hydrocarbons derived from petrochemical products, giving a more environmentally friendly choice to commonly used normal phase solvents, e.g., hexane, heptane, or chlorinated solvents. In addition, supercritical CO₂ has low viscosity, which allows high flow rates and faster separations, and has high diffusivity resulting in more efficient separations, reduced organic solvent consumption, and reduced cost in waste disposal, since CO₂ has a low environmental impact.⁵³ However, because of its non-polar character, it is not possible to employ pure CO₂ in samples containing polar analytes. In such cases, the addition of organic modifiers such as methanol or ethanol is necessary. The use of CO₂ with a higher percentage of organic modifiers can be referred to as enhanced fluidity liquid chromatography (EFLC), which has similar advantages to SFC. Following this, Lu *et al.* developed a fast, efficient, and green SFC method for the separation of actinomycin D and X₂ within less than 25 minutes. The proposed method allowed a reduction in the consumption of hazardous organic solvents since CO₂ and ethanol (80:20) were used as mobile phase instead of water and acetonitrile and the analysis time was also reduced by approximately 42% compared to the reference method presented in the study.⁵⁴

Another green alternative is micellar liquid chromatography (MLC), an RPLC mode with a mobile phase consisting of an aqueous surfactant solution above its critical micellar concentration (CMC). The idea of using aqueous pure micellar solutions as mobile phases in RPLC is very attractive due to lower cost and toxicity, and reduced environmental impact. In practice, however, the addition of a small amount of organic solvent to the micellar solution is necessary to achieve retention in practical time windows and improve peak efficiency and resolution. For this reason, MLC methods have become a trending topic in recent years when it comes to GAC.^{55,56,57} As an example, Ramezani *et al.* reported an eco-friendly MLC method using

a NADES solvent as a modifier of the mobile phase for melamine analysis in milk. The developed MLC revealed that the modified mobile phase composed of sodium dodecyl sulfate, NADES, and glacial acetic acid, significantly reduced the peak broadening and the melamine retention time resulting in an increased chromatographic resolution. In addition, the matrix effects analysis shows that the proposed method has the potential for direct injection analysis of the diluted milk samples.⁵⁸ The green potential of MLC methods is further highlighted by the study of Al-Shaalan *et al.* in which a modified green MLC method was used for the determination of residues of imidocarb dipropionate in food samples employing a NADES solvent as a modifier and direct sample injection. Furthermore, this study showed a complete evaluation of the greenness of the proposed method and the obtained reference methods using the Complex GAPI, AGREE, and RGB model tools. Comparing the results of the evaluations, the proposed method was superior to all the reference methods presented, with values of 0.78 for AGREE and 90.8 for the RGB model.⁵⁹

Multidimensional chromatography

The use of multidimensional chromatography can be considered a green strategy if used with green modulators. There are two types of multidimensional chromatography, the comprehensive one (GCxGC or LCxLC) in which all eluent from the first columns is transferred to the second column, and the heart cutting (GC-GC or LC-LC) in which only some fractions of the eluent are transferred from the first to the second column.⁶⁰ In two-dimensional chromatography, two orthogonal chromatographic columns are used, connected by a modulator, which transfers the eluent from the first to the second column.⁶¹ Due to the presence of two columns, it is possible to obtain a better resolution and lower detection limits with analysis time and reagent consumption similar to one-dimensional chromatography.⁶² Furthermore, due to the better peak capacity, the use of GCxGC or LCxLC allows for better separations even in complex samples. Although GCxGC is a relatively green technique, there is still the possibility of making it more environmentally friendly by replacing thermal modulators with flow modulators, which operate simply and cheaply and do not require the use of cryogenic gases.⁶³ However, the use of flow modulators leads to low sensitivity due to the limited modulation period.⁴⁴ The advantages of using the LCxLC include the prevention of sample loss and contamination and the possibility of automation. On the other hand, the volume of data generated can be large and its treatment requires chemometric tools as well as the optimization of operating conditions.⁶⁴ In addition, the cost of multidimensional chromatographic systems is frequently higher than that of one-dimensional chromatography.⁶⁵

Miniaturization of chromatographic systems

The application of a miniaturized system is usually related to the reduction of consumables, energy, and reagents needed to perform the analysis. The reduction of column dimensions and particle size reduces mobile phase flow rate and so the solvent or gas consumption. Miniaturization results in lower costs and lower production of waste, being more cost-effective and eco-friendlier. It is also possible to perform faster analysis and improve the sensibility of the method because the analytes are less soluble in the mobile phase. This strategy is very useful in forensics sciences and biomedicine, where large sample volumes are not always available. The miniaturization of all parts of a chromatographic system can result in portable systems which can be applied online, at-line, and on-site analysis, making the method greener.

The miniaturization of GC systems enables rapid in situ analysis of volatile organic compounds (VOC) for environmental protection, industrial monitoring, and toxicology.⁶⁶ The Lab-on-a-chip is another miniaturization approach that can be applied to chromatography systems. These miniaturized systems are manufactured on a chip platform (microfluidic devices) and have unique advantages including low maintenance cost, large-scale manufacturing, fast analysis, the need for very small amounts of solvents and samples, high-resolution detection, and excellent portability.⁶⁷ Recent advances related to sustainability in GC have focused on the miniaturization of specific pieces of equipment such as the preparation of micro columns and micro detectors. This field was made possible by parallel advances in certain processes and technologies of additive manufacturing, also known as 3D printing, microelectromechanical systems

(MEMS), lithography, and etching techniques.⁶⁸ Hsieh and Kim demonstrated the separation of a pair of structural isomers (isopentane and pentane) in a μ GC system with a circulatory loop consisting of two 25 cm open tubular micro columns while operating under a minimum pressure of <10 kPa available in current technology of chip-scale micropump. This demonstration was possible by extending the column length from 0.5 m (two 25 cm columns) to 12.5 meters, corresponding to 25 cycles using the circulatory column system.⁶⁹

The achieved effective column length of 12.5 meters is the longest ever used by any μ GC system and presented an alternative to the limitations of chromatography columns related to this parameter in μ GC. Furthermore, the described microscale system enabled rapid and sustainable analysis of complex samples with minimal sample volume and without entailing additional energy and carrier gas consumption, comprising GAC principles 2, 5, and 7. Despite recent developments in 3D printing, the peaks showed broadening due to the 0.6 μ L dead volume at the valve connections affecting the separation resolution. Thus, advances in this field are needed to develop instruments free of these limitations.

In another study employing a microfabricated gas chromatograph, Whiting *et al.* described the development and evaluation of a comprehensive two-dimensional chromatography (μ GC x μ GC) microsystem consisting of micro columns, a flame ionization detector (FID), and a nanoelectromechanical resonator system (NEMS) also used as a detector.⁷⁰ With this system, it was possible to separate a mixture of 29 polar compounds covering a boiling point range from 46 to 253 °C on a pair of microfabricated columns using a Staiger valve manifold in less than 7 seconds. Thus, the μ GC x μ GC-NEMS system allowed ultra-fast analysis with low energy and carrier gas consumption due to the use of greener modulators such as the pneumatic stop-flow modulator, showing that the two strategies employed in combination, miniaturization, and two-dimensional chromatography, made the method greener by meeting some of the principles of GAC.

To overcome the above-mentioned obstacles, Li *et al.* reported the development and characterization of a microfabricated column containing phosphonium ionic liquid (μ L) as the stationary phase and demonstrated the separation of polar and non-polar compounds using this column by analyzing alcohols, chloroalkanes, aromatics, aldehydes, fatty acid methyl esters, and alkanes.⁶⁶ The use of IL as the stationary phase provided robustness to the column concerning the presence of oxygen, humidity, and temperature, allowing the use of synthetic air as carrier gas and high temperatures during the separations. Consequently, faster and more sustainable separations were obtained without the need for additional accessories.

The reduction in analysis time is not only restricted to miniaturizations, Fialkov *et al.* obtained separations with complete analysis cycle times of less than 1 minute by employing for the first time LP-GC coupled to MS with low thermal mass resistive heating due to the use of LTM technology for the rapid increase temperature and cooling of the capillary column.⁷¹ An example of recent advances in LC miniaturization is the study carried out by Peretzki and Belder. In this study, the authors presented a chip-integrated approach to post-column segmentation of Normal Phase Liquid Chromatography (NPLC), obtained by integrating a chiral NP-chip-HPLC column, a Flow focusing droplets, and a segmented flow channel on a single glass microfluidic chip.⁷² This allowed continuous segmentation of the eluent into droplets that are collected and transported through a continuous immiscible phase. The combination of NP-chip-HPLC and droplet-based microfluidics also allowed fractionation and conservation of chromatographic runs for other picoliter-scale downstream processes. With this, the authors tested three microfluidic devices with different structural arrangements for the separation of the (R)- and (S)- trifluoro anthryl ethanol isomers under the same elution conditions (0.5 μ L min^{-1} n-heptane/2-propanol 95/5 (v/v)) and continuous phase (0.10 μ L min^{-1} ethylene glycol) and the sample volume inject was very low (5 μ L). The separation of the isomers took less than 5 minutes and the peak widths at half height were approximately 0.17 min (isomer R) and 0.22 min (isomer S) for all three devices. Furthermore, the efficiency was in the range of 27.400 to 41.600 for the three devices tested. The proposed system combined the advantages of minimized reagent consumption, dead volume-free interconnections, high separation speed and performance, and the possibility of integrating additional processes from chip-HPLC with the benefits of droplet-based microfluidics such as avoiding

peak distortion post column. As a result, the system complies with GAC principles and allows coupling with other detectors after chromatographic separation, expanding the application of the system.

CONCLUSIONS

The use of miniaturized, online, or at-line sample preparation techniques, or the use of green solvents is essential to improve the green aspects of chromatographic methods and to conform to GAC principles, especially for GC, where few modifications in separation conditions are possible.

The advances made in the miniaturization of chromatographic techniques have sought to overcome the limitations presented by system connections, microcolumn fabrication, and micro detectors. Such advances were possible due to the development of additive manufacturing techniques, but most published works present prototypes of micro chromatographic systems with many factors to be improved, such as band broadening due to the presence of large dead volume. Within miniaturization, LOC-based chromatographs are the most promising systems due to the potential to integrate multiple functionalities on a single chip, minimal reagent and power consumption, fast separations and good performance, and the possibility of manufacturing these systems on a large scale, resulting in low manufacturing cost.

However, more methods should be developed to evaluate which applications are possible and if they are repeatable, and how durable they are as instruments, since the low cost of large-scale production may become an environmental problem if the durability of these systems is short. In addition, no metrics were applied in the miniaturization studies. The use of methods and techniques that use green solvents as a mobile phase, such as MLC and SFC, for example, should be further explored for the replacement of traditional LC methods. As these methods drastically reduce the environmental and occupational hazards related to the use of organic solvents and consequently make the method more economical.

Most papers that mentioned the development of greener methods did not evaluate the methods or evaluated the methods using qualitative metrics. It is important to consider that the green features claimed in these approaches should be supported by suitable green metrics. The AGREE algorithm is the most suitable metric for evaluating method greenness as it quantitatively covers the 12 principles of GAC. However, evaluating only the green aspect of the method is not sufficient for the method to be considered suitable for its intended use. Therefore, the developed methods must be evaluated by metrics that encompass the three pillars of sustainable development: the green aspects, analytical performance, and economic viability as proposed by the RGB 12 metric.

Conflicts of interest

The authors declare no conflict of interest of any kind.

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