

Resource management in HPLC: Unveiling a green face of pharmaceutical analysis

Jevrem Stojanović*, Jovana Krmar, Biljana Otašević, Ana Protić

University of Belgrade – Faculty of Pharmacy, Department of Drug Analysis,
Vojvode Stepe 450, 11010 Belgrade, Serbia

*Corresponding author: Jevrem Stojanović; e-mail: jstojanovic@pharmacy.bg.ac.rs

Abstract

High-pressure liquid chromatography (HPLC) is a technique of paramount importance in the analysis of pharmaceuticals because of its ability to separate moderately polar to less polar compounds, such as drugs and related substances. The concept of green analytical chemistry (GAC) aims to provide more environmentally friendly and safer analytical methods in terms of reagents, energy, and waste. One of the major challenges of GAC is to find an appropriate approach to evaluate the greenness of analytical methods. An extension of GAC, called white analytical chemistry (WAC), has been introduced to consider not only environmental friendliness, but also other aspects that contribute to the sustainability of methods, such as analytical and economic or practical efficiency. HPLC methods are intrinsically not green, due to the high consumption of toxic organic solvents and the resulting generation of large amounts of toxic waste. Fortunately, there are many approaches to overcome the non-green character of HPLC methods. In this article, various modifications of the HPLC methods that increase its environmental friendliness are presented, as well as the various tools used to evaluate environmental friendliness. In addition, the new concept of white analytical chemistry is presented.

Key words: high-performance liquid chromatography, greenness assessment, sustainability

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Introduction

High-performance liquid chromatography (HPLC) is one of the most commonly used analytical techniques in the analysis of pharmaceuticals. To date, HPLC, especially in the reversed-phase mode (RP-HPLC), has proven to be almost irreplaceable in this field, due to its ability to adequately retain and separate moderately polar and less polar compounds such as drugs, their impurities, degradation products, and metabolites (1). The desired chromatographic behaviour of drugs can be achieved by careful selection of the chromatographic column, adjustment of the content of organic modifiers in the mobile phase, column temperature, flow rate of the mobile phase, etc. In addition, various additives can be added to the mobile phase in order to improve retention behaviour and peak shape or to achieve chiral resolution (2). Various physicochemical detection principles can be applied so that analytes without specific properties (e.g., chromophores) can be easily detected. Some examples are refractive index detector (RI), mass spectrometer (MS), corona charged aerosol detector (CAD), and evaporative light scattering detector (ELSD) (3).

Green chemistry is a concept primarily aimed at reducing or avoiding the use and production of substances that pose a risk to human health or the environment, as defined by Anastas (4). Since its emergence in the early 1990s, green chemistry has been dedicated to organic synthesis, as it is recognized as an area of chemistry characterized by the consumption and production of large quantities of hazardous substances (5). When the concept of green analytical chemistry (GAC) was introduced in 1999, the scope of green chemistry was extended to analytical chemistry (4, 6). The introduction of GAC led to a broader understanding of the need to minimize the adverse environmental impact of analytical procedures. According to the principles of green chemistry, analytical chemists are responsible not only for providing reliable analytical results, but also for developing more environmentally friendly analytical procedures in terms of reagent consumption, energy consumption, and waste generation and management.

HPLC is essentially not an environmentally friendly analytical technique because it has a high consumption of toxic organic solvents as mobile phase eluents and consequently generates large amounts of waste (7, 8). The most commonly reported values for the daily waste generated by a conventional HPLC system range from 1 to 1.5 L, which is a considerable amount when multiplied by all the instruments present in a laboratory (8–11). Some reports estimate the total amount of HPLC waste per year to be about 34 million litres (12, 13). Sample preparation, along with chromatographic separation, is one of the most demanding steps in terms of reagent consumption, energy consumption, and waste generation, and is usually considered a critical step for the environmental friendliness of the entire analytical procedure (5, 14). Samples to be analyzed by HPLC are usually not "clean" enough to be injected directly without sample preparation. The required sample preparation step also contributes significantly to the environmental impact of HPLC methods. Fortunately, despite the inherent non-greenness of HPLC methods, there are a plethora of different aspects of the analytical procedure that can be significantly improved with respect to the greenness of the method (8, 9).

Various metrics and tools have been proposed in the literature to assess greenness of the analytical methods (5, 15–17). It is important to note that none of these approaches is ideal, so we need to understand their features, advantages and disadvantages in order to choose an appropriate tool.

The sustainable analytical method is not only green, but also practical, economical and reliable for the intended application. In order to simultaneously evaluate all the characteristics that contribute to the sustainability of the method, the concept of "white analytical chemistry" has recently been introduced (18).

The aim of this article is to summarize different approaches to greening HPLC methods. The basic principles of GAC are presented, as well as various metrics for evaluating the greenness of analytical methods, and basic ideas, know-how, their advantages and limitations are explained. Finally, the recently introduced term "white analytical methods" is discussed.

Green analytical chemistry principles

For a long time, green analytical chemistry lacked detailed principles which would serve as a guide for embedding greenness in analytical methods. The 12 principles of green chemistry proposed by Anastas and Warner (19) are generally not directly reflected in analytical methods, with the exception of some principles, e.g., on waste prevention (Principle 1), safer solvents and reagents (Principle 5), energy efficiency (Principle 6), and avoidance of derivatization (Principle 8) (20). To facilitate the implementation of green chemistry by analytical chemists, Galuszka et al. proposed 12 GAC principles and the mnemonic SIGNIFICANCE shown in Figure 1 and Figure 2 (20). The proposed GAC principles are clear, all-inclusive, and therefore suitable for green chemistry implementation and greenness assessment in chemical analysis area. The following is a brief discussion of the principles of GAC.

Principle 1 states that direct analytical measurement should be performed in order to avoid sample preparation step (20). Such an approach eliminates the need for sample preparation, which typically involves the consumption of toxic solvents, reagents, and energy, and offers the possibility of direct in-situ measurement. Direct measurement significantly reduces the number of analytical steps required and the overall duration of the analysis. However, the characteristics of the analytical technique, the complexity of the sample matrix, and the nature of the analyte prevent the method from meeting this principle.

Principle 2 states that minimal sample size and minimal number of samples are the goals (20). A smaller sample size results in less waste, which is highly desirable. Statistics for selecting sampling sites and the use of non-invasive field screening methods can result in a smaller number of samples (16, 20). Miniaturized methods can provide reliable results even with a significantly reduced sample size. However, it is important to remember that too small a sample size or number can lead to unreliable analytical results because sample representativeness might be lost (20).



Figure 1. The 12 principles of GAC (adapted from reference (20))

Slika 1. Dvanaest principa GAC (predstavljjanje prilagođeno iz reference (20))

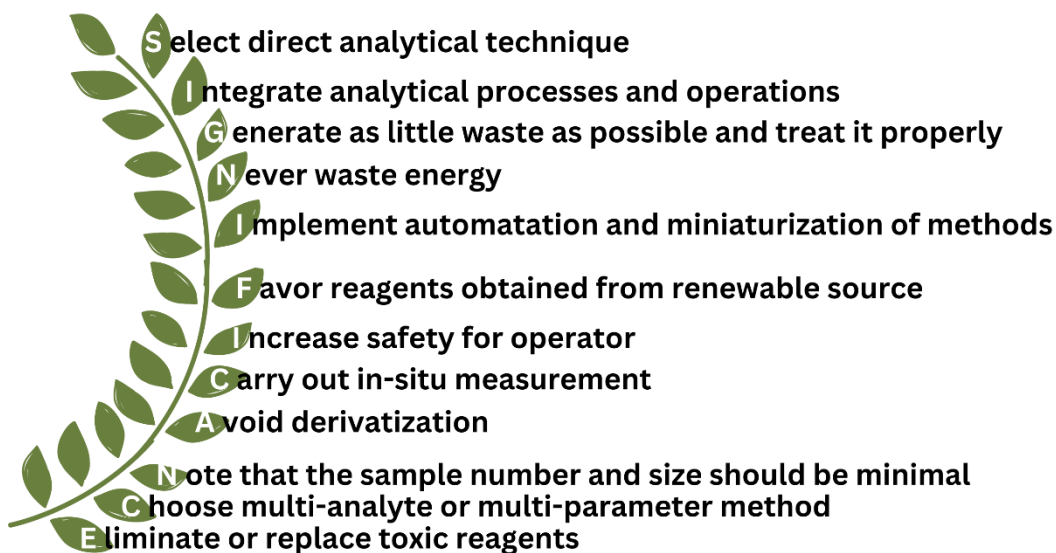


Figure 2. SIGNIFICANCE mnemonic adapted from reference (20)

Slika 2. Akronim SIGNIFICANCE kao slikoviti podsetnik na principe zelene hemije (prilagođeno iz reference (20))

Principle 3 states that measurements should be made in situ, i.e., the instruments should be as close as possible to the sampling location (20). This eliminates costly and time-consuming transportation, which contributes to air pollution, preservation, which consumes reagents and/or energy, and sample storage, which also consumes energy. Sample preparation is eliminated or minimized. Four possible scenarios in terms of distance between the instrument and the sampling site: in-line, on-line, at-line, and off-line sampling are explained in the reference (8).

Principle 4 states that integration of analytical processes saves energy and reduces the use of reagents (20). In general, the greater the number of steps in an analytical procedure, the more reagents, energy and time are required for analysis. Integration can reduce the number of individual steps in the analytical procedure, resulting in corresponding savings.

Principle 5 states that automated and miniaturized methods should be selected (20). Miniaturized methods require a smaller amount of sample, smaller amount of reagents, and lower energy, while automation increases operator safety (16).

Principle 6 states that derivatization should be avoided (20). Derivatization requires the use of reagents and time and usually results in the generation of toxic waste. However, sometimes derivatization is necessary to improve analyte response or extraction (16). In such cases, derivatization should be integrated, or safer, more environmentally friendly reagents should be used (20).

Principle 7 states that generation of a large volume of analytical waste should be avoided and proper management of analytical waste should be provided (20). The amount of waste can be reduced by reducing the number of steps in the analytical procedure or the amount of solvents and reagents used. Ideally, analytical waste should be treated by recycling, degradation, or passivation to reduce its toxicity (20).

Principle 8 states that multianalyte or multiparameter methods are preferred versus methods using one analyte at a time (20). This principle is very important when complex samples are analyzed. In this case, a single analyte method would require a greater number of runs, a greater amount of solvents and reagents, and would not be time-efficient.

Principle 9 states that the use of energy should be minimized (20). Energy is consumed not only for sample preparation and analytical measurement, but also for transportation, preservation, and storage.

Principle 10 states that reagents obtained from renewable sources should be preferred (20). Increased use of reagents from renewable sources can conserve non-renewable sources (20).

Principle 11 states that toxic reagents should be eliminated or replaced (20). Toxic reagents pose a risk to the environment and the user. Whenever possible, more environmentally friendly alternatives should be used.

Principle 12 states that the safety of the operator should be increased (20). Volatile toxic reagents, as well as flammable, explosive, and corrosive reagents, can endanger operator safety. Ideally, such reagents should be replaced. Automation, miniaturization and on-line waste decontamination can also have a positive impact on operator safety (20).

Approaches to the greening of high-pressure liquid chromatography

The environmental friendliness of HPLC methods is primarily compromised by the use of large amounts of hazardous and toxic organic solvents such as acetonitrile or methanol. Acetonitrile is considered the "gold standard" in RP-HPLC analysis because it has the desired physicochemical properties, such as miscibility with water, the main component of the mobile phase in RP-HPLC systems; the low viscosity of water/acetonitrile mixtures, which results in lower backpressure and allows higher mobile phase flow rates; the low boiling point, which allows the use of aerosol-based detectors; and the low UV cut-off (190 nm) (7, 9, 11). In addition, acetonitrile is chemically inert and is unlikely to react with analytes in the chromatographic column and lead to the formation of in situ degradation products. The high eluotropic strength of acetonitrile provides adequate retention time and total run time when sample mixtures contain highly lipophilic components that are strongly retained by the stationary phase. Another commonly used organic modifier in RP-HPLC is methanol. Methanol is also a water-miscible solvent with a low UV cut-off (205 nm), lower solvent strength, and in some cases different selectivity than acetonitrile (21). Methanol is inferior to acetonitrile because its mixtures with water have higher viscosity, resulting in higher backpressure and allowing lower mobile phase flow rates.

Unfortunately, acetonitrile and methanol are highly flammable, volatile, and labelled with the signal word "Danger" according to the Globally Harmonised System of Classification and Labelling of Chemicals (GHS) (5, 10). Both of the above solvents may cause acute and chronic toxicity to humans and aquatic life (9). The waste generated from the use of acetonitrile and methanol must be properly disposed of, which involves additional costs for laboratories (10).

The ultimate objective of all approaches to developing green HPLC methods is therefore to reduce or, if possible, eliminate the use of toxic organic solvents in sample preparation or chromatographic separation (8–10). The different approaches are summarized in Figure 3.

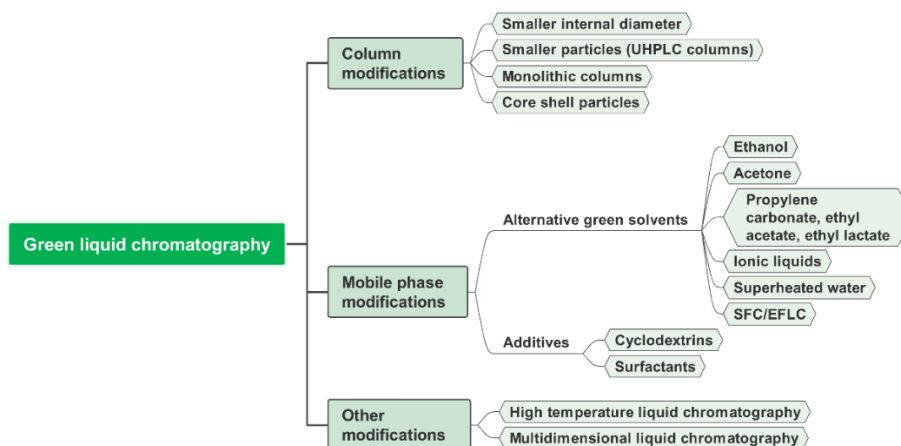


Figure 3. Approaches to the greening of liquid chromatography

Slika 3. Pristupi u povećanju ekološke prihvatljivosti tečne hromatografije

Column-related modifications

One of the simplest modifications in HPLC is the selection of columns with a small internal diameter (i.d.) (8–10). To achieve a similar retention time of the analytes on columns with a smaller i.d. than that of the column originally used, the flow rate of the mobile phase should be reduced according to the equation:

$$F_S = F_L \left(\frac{r_S}{r_L} \right)^2 \quad (1)$$

where F_S and F_L are flow rates in column with a smaller i.d. and larger i.d., and r_S and r_L are diameters of the smaller and larger column. For example, conventional columns with an internal diameter of 3-5 mm require flow rates of 0.3-1 mLmin⁻¹, while capillary columns with an internal diameter of 0.1-1 mm require much lower flow rates of 0.4-200 μ Lmin⁻¹ (8). In this way, a similar run time can be achieved with lower flow rates and consequently lower mobile phase consumption and waste generation. Another major advantage of this approach is the improvement in sensitivity, as the analytes are diluted in smaller volumes of mobile phase. A limitation of this approach is that column efficiency may be deteriorated due to the extra-column band broadening if HPLC instruments have excessive extra-column volume relative to column volume. While conventional (i.d. 3-5 mm) and narrow-bore (i.d. 2.1-3 mm) columns can be used with conventional instruments, micro-bore (i.d. 1-2 mm), capillary (i.d. 0.1-1 mm), and nanocapillary (i.d. 0.025-0.1 mm) columns require instruments with special mobile phase pumps. A significant improvement in the sensitivity of the method provides an opportunity to collect and analyze small amounts of the sample, especially with capillary and nanocapillary columns (8,9). Some examples of the use of small internal diameter columns in drug analysis can be found in the literature (22–30).

Another, more environmentally friendly variant of HPLC is ultra-high pressure liquid chromatography (UHPLC) (8–10). UHPLC columns consist of a stationary phase in the form of sub-2 μ m particles packed into short columns (e.g., 5 cm) with a relatively small internal diameter (e.g. 2.1 mm) (31, 32). The characteristics of UHPLC columns combined with low flow rates result in shorter run times, higher sample throughput, lower energy consumption, lower mobile phase consumption, and ultimately lower amount of waste produced during the chromatographic analysis. In addition, highly efficient separation is achieved in much less time than with conventional columns. Although backpressure is expected to decrease as column length is reduced, smaller particles in UHPLC columns result in an overall increase in backpressure (up to 1200 bar) (10). Therefore, special UHPLC equipment is required to withstand such a high pressure. As with the use of narrow columns, the instrument should have a sufficiently small extra column volume to avoid band broadening and loss of efficiency. The applications of UHPLC in pharmaceutical analysis are numerous (33–38).

Columns packed with superficially porous particles, also known as core shell or fused core particles, have shown comparable efficiency and separation speed to UHPLC columns (39). Core shell particles, as the name implies, consist of a solid inner core surrounded by a porous outer shell. Although the efficiency of such columns is lower than

that of UHPLC columns, they provide better efficiency than conventional columns due to lower mass transfer resistance, low internal porosity, small diffusion distances, and narrow particle size distribution (9,40). In addition, columns packed with core shell particles are superior to conventional columns in terms of speed of analysis. The advantage over UHPLC is that core shell particles generate much lower backpressure and can therefore be used with conventional HPLC instruments able to withstand backpressure up to 400 bar. Finally, core shell particles packed into short columns can not only provide fast and efficient separation, but also reduce mobile phase consumption (10). Due to the lower backpressure generated by the core shell particles, highly viscous ethanol/water mobile phases can be used, which are more environmentally benign compared to the commonly used acetonitrile/water mixtures (41). Further details on core shell particles and their application in drug analysis are summarized in the reference (42).

Monolithic columns are packed with macro- and mesoporous rod-shaped stationary phase. Such a structure allows the use of higher flow rates without high backpressure, thus speeding up the analysis (8–10,43). When monolithic columns with a small internal diameter are used, the consumption of organic solvents can be reduced, in addition to a shorter analysis time. Mobile phases with higher viscosity such as ethanol/water mixtures can be used in conjunction with monolithic columns (8). Some applications of monolithic columns for the development of environmentally friendly analytical methods are presented in the references (44–47).

Mobile phase-related modifications

Greener solvents

As mentioned earlier, most commonly used RP-HPLC solvents, such as acetonitrile and methanol, are not environmentally friendly and should be replaced with greener alternatives. Some solvent selection guides (SSGs) introduced by pharmaceutical companies can be helpful in selecting such alternatives (48–50). These guides evaluate the degree of environmental friendliness of various organic solvents based on environmental, health, and safety (EHS) criteria and life cycle analysis (LCA). From the point of view of analytical chemists, the disadvantage of SSGs is that they focus on solvents that are mainly used for organic synthesis, and many solvents that can be used in HPLC are not considered. Another interesting assessment of the environmental friendliness of solvents is based on the modified CHEMS-1 model (51). Total analytical hazard values (taHV) have been calculated for some organic solvents most commonly used in analytical laboratories. This approach for organic solvents is comprehensive as it considers various toxicological and ecotoxicological effects, as well as different types of exposure to solvents, including inhalation, which is the main exposure route for analysts in laboratories. In addition, this approach is very simple, as almost all input data can be taken from material safety data sheets. The calculated values can be useful in the selection of organic solvents during method development, and also in the evaluation of the greenness of solvents as part of the evaluation of the greenness of analytical methods. According to the CHEMS-1 model applied to evaluate the total analytical hazard value

(taHV) for various solvents commonly used in analytical laboratories, acetonitrile and methanol were assigned values of 26.8 and 15.7, respectively. These values indicate that acetonitrile and methanol are less hazardous than hydrocarbons and chlorinated hydrocarbons (e.g., the taHV for pentane is 36.4 and for chloroform is 103.8), but still significantly more hazardous than certain solvents (e.g., acetone and ethanol with a taHV of 2.6 and 7.2, respectively) (51). The results of this scoring are consistent with the known fact that more polar RP-HPLC solvents are less toxic than nonpolar NP-HPLC solvents, but also point to further opportunities to achieve greener RP-HPLC separations. Some of the greener alternatives to conventional RP-HPLC solvents are ethanol, isopropanol, n-propanol, acetone, propylene carbonate, ethyl lactate and ethyl acetate (48).

When it comes to the greener choice between acetonitrile and methanol, methanol may be a better choice due to its relatively lower toxicity, easier biodegradability, and lower disposal costs (8,9,48).

Ethanol is considered one of the greenest organic solvents with similar physicochemical properties to acetonitrile and methanol (9, 48). Ethanol is derived from natural sources, and is less toxic and volatile than acetonitrile or methanol. Waste generated by ethanol/water mixtures as mobile phases is considered environmentally safe, which significantly reduces disposal costs (10,48). The main problem with ethanol/water mixtures as mobile phases is their high viscosity, which leads to high backpressure (8,48). To overcome this limitation, separation can be performed using core shell particles or monolithic columns and/or at elevated temperature. Another option is to switch to UHPLC instruments that can tolerate high backpressure. Other disadvantages include lower eluotropic strength and a slightly higher UV cut-off value (210 nm) compared to acetonitrile, resulting in higher noise and lower sensitivity (10,48). Although ethanol is known to have lower chromatographic performance than acetonitrile or methanol, many articles have demonstrated satisfactory separation of analytes, especially with modern column technologies and at elevated temperatures, as shown in the following references (7,10,48).

Acetone may be a suitable alternative, being at the same time less toxic, readily biodegradable, and desirable because of its physicochemical properties and the resulting chromatographic performances, which are very similar to those of acetonitrile (8,9,48). Acetone is characterized by a higher eluotropic strength than acetonitrile, which offers the possibility of achieving the desired retention time of the analyte with a lower acetone content in the mobile phase, and consequently lower acetone consumption (7). However, these advantages are also counterbalanced by some disadvantages, including its high UV cut-off value (340 nm), which limits the use of acetone/water mixtures as mobile phases in HPLC instruments equipped with UV or photodiode array (PDA) detectors (8,9,48). Given the widespread routine use of UV/PDA detectors, this can be a serious obstacle. Nevertheless, acetone can be successfully used as an organic modifier when detection of the analyte is performed with mass spectrometers or aerosol-based detectors such as a charged aerosol detector (CAD) (7,52–54).

Solvents such as propylene carbonate, ethyl acetate, and ethyl lactate are environmentally friendly, inexpensive, and readily biodegradable, but their use is severely hampered by their drawbacks (48). Propylene carbonate has high viscosity and is difficult to mix with water, so a third component such as methanol or ethanol must be added to the mobile phase (9). Ethyl acetate and ethyl lactate are particularly susceptible to degradation under acidic or alkaline conditions and have high UV cut-off values (48).

Ionic liquids (ILs) are molten salts composed of organic cations and organic or inorganic anions that remain liquid at ambient temperature (9,48). Interest in ILs stems from their advantages such as miscibility with water and organic solvents, reusability, ability to dissolve organic and inorganic compounds, low vapor pressure, and non-flammability. ILs are typically used in small amounts as mobile phase additives, i.e., as silanol blockers, to improve the peak shape of cationic analytes. Another application is the addition of larger amounts of ILs to the aqueous mobile phase as a substitute for toxic organic solvents. Nevertheless, the environmental friendliness of ILs is questionable, as they are known to be a hazard to aquatic systems. Another disadvantage is their high price (9, 48).

HPLC methods using completely aqueous mobile phase are the greenest solution because water as a solvent is neither toxic nor hazardous (9,48). Nevertheless, there are serious limitations with this approach. The biggest problem with water as a mobile phase is the inability to elute lipophilic organic compounds that are strongly retained by the stationary phase. This problem can be partially solved by using water heated above its boiling point but below its critical point (374°C) under elevated pressure. Water at such a high temperature has much lower polarity and much higher elution strength. This technique is referred to as superheated water chromatography (SHWC). Problems with SHWC include instrumental requirements, the need for special columns that are temperature resistant and compatible with 100% aqueous mobile phases, and possible degradation of the analyte during chromatographic separation (9,10,48).

The chromatographic technique using carbon dioxide under pressure as the mobile phase is called supercritical fluid chromatography (SFC). Carbon dioxide is considered a green solvent because it is non-toxic, non-flammable, non-explosive, and has low disposal costs (9). Carbon dioxide is not necessarily in a supercritical state, but still possesses certain properties of supercritical fluids, such as low viscosity and high diffusivity similar to gasses, and high density and solubilizing power, similar to liquids. These properties contribute to very fast and highly efficient separations. The polarity of carbon dioxide is similar to that of hydrocarbons, which means that carbon dioxide cannot elute polar compounds. Therefore, mobile phases usually contain polar organic solvents such as methanol or ethanol as modifiers (up to 40%) (10). SFC is commonly used in analytical and preparative chiral chromatography, but may also be advantageous in achiral chromatography. From a GAC perspective, SFC offers the advantages of very fast analysis, lower organic solvent consumption, and little or no toxic waste, e.g., when pressurized carbon dioxide is combined with ethanol (8, 10). The amounts of carbon dioxide emitted by the instruments do not contribute significantly to its atmospheric

levels. Special instruments with backpressure regulators are required for SFC to prevent pressure reduction and associated phase separation. Current instrumentation development may promote the application of SFC.

Enhanced fluidity liquid chromatography (EFLC) uses organic solvents or hydro-organic mixtures enriched in high levels of pressurized carbon dioxide as the mobile phase (8, 9). The mobile phase used in EFLC retains the desirable physicochemical properties of both supercritical fluids (high diffusivity, low viscosity) and liquids (elutropic strength). As with SFC, pressure changes can be used to alter density, polarity, and solvent strength, but lower pressures are generally required to prevent phase separation compared to SFC. Other advantages include high optimal linear velocity, high efficiency, shorter analysis time, and the ability to use long capillary columns (8, 9). Because polar organic solvents such as ethanol are used, this technique is suitable for the separation of moderately polar to polar compounds. A particularly highlighted application of EFLC is enhanced fluidity hydrophilic interactions chromatography (EFL-HILIC). Satisfactory EFL-HILIC separations, much greener than conventional HILIC separations, have been performed by replacing acetonitrile/water with methanol/water or ethanol/water mobile phases enriched in certain proportions of carbon dioxide, allowing efficient separations in a relatively short time (55,56).

Mobile phase additives

Increasing the number of chemicals used in the analysis seems unreasonable if we want to develop a green HPLC method. However, small amounts of some mobile phase additives have been shown to reduce the amount of organic modifiers required to elute the analytes, thereby shortening run times without producing toxic and hazardous wastes or causing health or safety problems for the operator. Examples of such additives include surfactants and cyclodextrins (CDs).

Micellar liquid chromatography (MLC) is a system based on RP-HPLC that uses an aqueous solution of surfactants at concentrations above the critical micellar concentration (CMC) as the mobile phase. Micelles in the mobile phase, as well as adsorbed surfactant molecules, introduce additional partition equilibria that modulate the retention behaviour of the analytes (2). The retention time of binding analytes is significantly reduced in the MLC. A purely aqueous solution of a surfactant rarely allows the elution of lipophilic compounds within a reasonable run time. In such cases, separation at higher temperatures or the addition of organic modifiers may be an appropriate solution (2). Environmentally friendly aspects of MLC include safe, non-flammable, non-toxic or less toxic mobile phases that contain no or only small amounts (3-15%) of organic modifiers (9,48). In addition, commonly used modifiers such as propanol or butanol are more environmentally friendly than acetonitrile and methanol. The waste produced by MLC separations is considered non-hazardous because the surfactants are readily biodegradable (9, 48, 57). In addition, the tedious, solvent-requiring, and time-consuming sample preparation normally required in bioanalysis can be avoided because micelles are able to solubilize proteins from biological fluids and

prevent their irreversible adsorption to the stationary phase and consequent column plugging (57).

Cyclodextrins (CDs) are cyclic oligosaccharides with six to eight glucose units linked to form a frustoconical cylindrical molecule. CDs are characterized by a hydrophilic outer surface, which provides their water solubility, and a hydrophobic inner cavity, which can form inclusion complexes with hydrophobic organic compounds (2). The number of glucose units determines the size of the hydrophobic cavity, which in turn limits the size of organic molecules that can be incorporated into the cavity. β -CD with seven glucose units has sufficient cavity size to accommodate heterocyclic and aromatic compounds such as active pharmaceutical ingredients and related substances (2). This makes β -CD particularly suitable for various pharmaceutical applications, including their use as mobile phase additives. CDs, like surfactants added to the mobile phase, form a pseudophase, thus creating additional partition equilibria. Organic molecules can be partially or completely incorporated into the cavity of CDs, forming more or less stable inclusion complexes. The stability constant of the inclusion complexes significantly influences the retention behavior of the analytes to be separated (58). As mobile phase additives, CDs can enable chiral separation based on differences in the stability of inclusion complexes formed with different enantiomers (59,60). In addition to applications in chiral analysis, CDs are recognized as additives that provide some green opportunities in RP-HPLC. Considering that the formation of inclusion complexes of CDs with analytes increases the solubility of analytes in water, one might expect that mobile phases modified with CDs can elute analytes from the column in a shorter time, and also with a significantly lower amount of organic modifiers in the mobile phase (2). Shorter analysis times, lower consumption of organic modifiers, and their replacement with greener alternatives will jointly lead to separations that are cost-, time-, and energy-efficient and generate less toxic or non-toxic waste (61). CDs are non-toxic, nature-based products derived from renewable sources (starch). They are therefore perfect as mobile phase additives providing undeniably greener chromatographic separations. CDs are UV transparent, which allows their use in HPLC-UV/PDA systems, but their compatibility with LC-MS is limited. CDs are only weakly adsorbed to the stationary phase and are therefore easily washed out (2).

Other modifications

Temperature is the most easily manipulated parameter during HPLC method development (8). The term high temperature liquid chromatography (HTLC) is used to refer to separations performed at temperatures higher than ambient but lower than the critical temperature (9). The elevated temperature during separation provides numerous advantages, including lower viscosity and backpressure, which allows for a higher mobile phase flow rate and shorter run time; lower polarity and higher eluotropic strength of ethanol and water, which allows their use as the mobile phase; and, typically, a shorter retention time of the analytes (8). Due to the lower mass transfer resistance resulting in higher efficiency and possible changes in selectivity, overall chromatographic

performance can also be improved at an elevated temperature. However, elevated temperatures require certain instrumental adjustments and stable stationary phases, and are not suitable when analytes are susceptible to thermal degradation (8,9).

When analyzing complex samples, it may be impossible to separate all components in a single chromatographic run using one-dimensional liquid chromatography (1D-LC) (8). Therefore, one approach is to perform multiple chromatographic runs to obtain the desired information. Such an approach requires a long total time for analysis, a high consumption of organic solvents, and a high waste generation. The other approach, which is wiser from the GAC point of view, is two-dimensional liquid chromatography (2D-LC). 2D-LC is a chromatographic system that uses two different separation mechanisms to achieve the required peak capacity when analyzing complex samples. All peaks are separated in a single run, resulting in significantly shorter analysis time and thus savings. Additional improvement in analysis speed can be achieved by using high temperatures and shorter columns. 2D-LC allows the use of green solvents with high UV cut-off values such as acetone in the first dimensions, which has a positive effect on the environmental friendliness of the method (8).

Sample collection, sample preparation, automation, and waste management are also very important factors in the environmental impact of HPLC methods. However, these issues are beyond the scope of this paper and are discussed in detail elsewhere (8,9,20).

Greenness assessment

National Environmental Method Index (NEMI)

The National Environmental Method Index (NEMI) is a searchable database of methods for environmental analysis (62). The results of a search by analyte name are displayed in the form of a table containing all analytical methods provided for that analyte. For each method, a comprehensive description is given, allowing comparison of the methods in terms of performance required for the intended purpose, such as accuracy, detection limit, precision, etc. In addition, NEMI has provided a greenness assessment tool in which a greenness profile is presented in the form of a pictogram with four quadrants that relate to different acceptance criteria, as shown in Figure 4. Acceptance criteria relate to: (i) PBT (persistent, bioaccumulative, toxic); (ii) hazardous; (iii) corrosive; and (iv) waste. The criteria are not met if: (i) PBT chemicals are used; (ii) hazardous chemicals are used; (iii) the pH during analysis is less than 2 or greater than 12; and (iv) the amount of waste is greater than 50 g (62). The quadrant corresponding to the unmet criterion remains unfilled. Otherwise, we say that the criterion is met and fill in the corresponding quadrant with green color. Chemicals are classified as PBT or hazardous only if they are included in specific lists, e.g. EPA's TRI. The NEMI symbol is practical because it is easy to read and understand. On the other hand, the NEMI symbol is an old tool, dating from the time when no GAC principles existed. The acceptance criteria used for greenness assessment by NEMI are based only on the chemicals used, while other important aspects of analytical methods such as energy consumption, sample collection, transportation, etc. are not considered. In addition, this approach is only

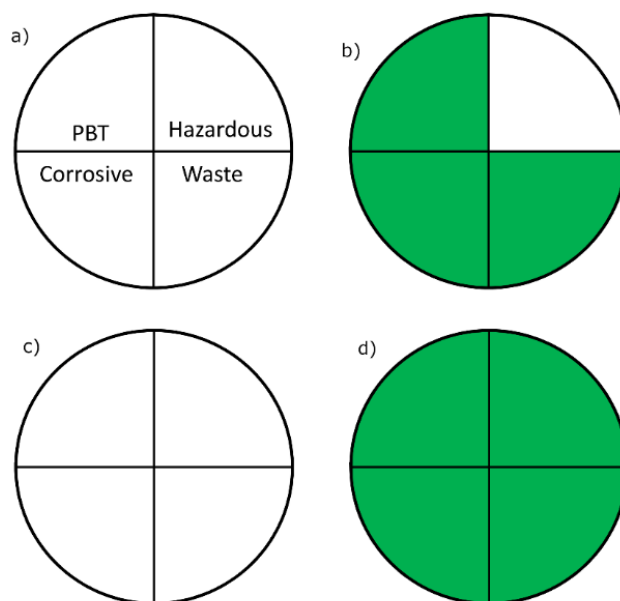


Figure 4. NEMI symbols: a) quadrants corresponding to the different evaluation criteria; b) for methods using hazardous chemicals; c) for methods not meeting any evaluation criteria; and d) for methods meeting all evaluation criteria.

Slika 4. NEMI simboli: a) kvadranti koji odgovaraju različitim evaluacionim kriterijumima; b) za metode koje koriste opasne hemikalije; c) za metode koje ne ispunjavaju ni jedan kriterijum po kome se procenjuju kao ekološki prihvatljive; d) metode koje su ekološki prihvatljive po svim evaluacionim kriterijumima.

qualitative and the creation of symbols requires a review of specific lists of PBT and hazardous substances, which can be time consuming (15).

Green Analytical Procedure Index (GAPI)

The Green Analytical Procedure Index (GAPI) is a recently introduced semi-quantitative tool for evaluating the environmental friendliness of analytical methods (15). The main advantage of this tool is its completeness, as it considers all steps of the analytical procedure from sampling to sample analysis. GAPI uses pictograms consisting of five pentagrams, each of which corresponds to a specific step of the analytical procedure. A GAPI pictogram is presented in reference (15).

The greenness of each step of the analytical procedure is evaluated and a corresponding color is assigned to the corresponding field in the GAPI pictogram (15). Three colors are used: green - for the greenest possible approach, yellow - for a less green approach, and red - for the least green approach. For details on the color assignments, see Figure 5. If some steps in the analytical procedure are not required (e.g., sample preparation for direct analytical methods), the corresponding pentagram in the pictogram

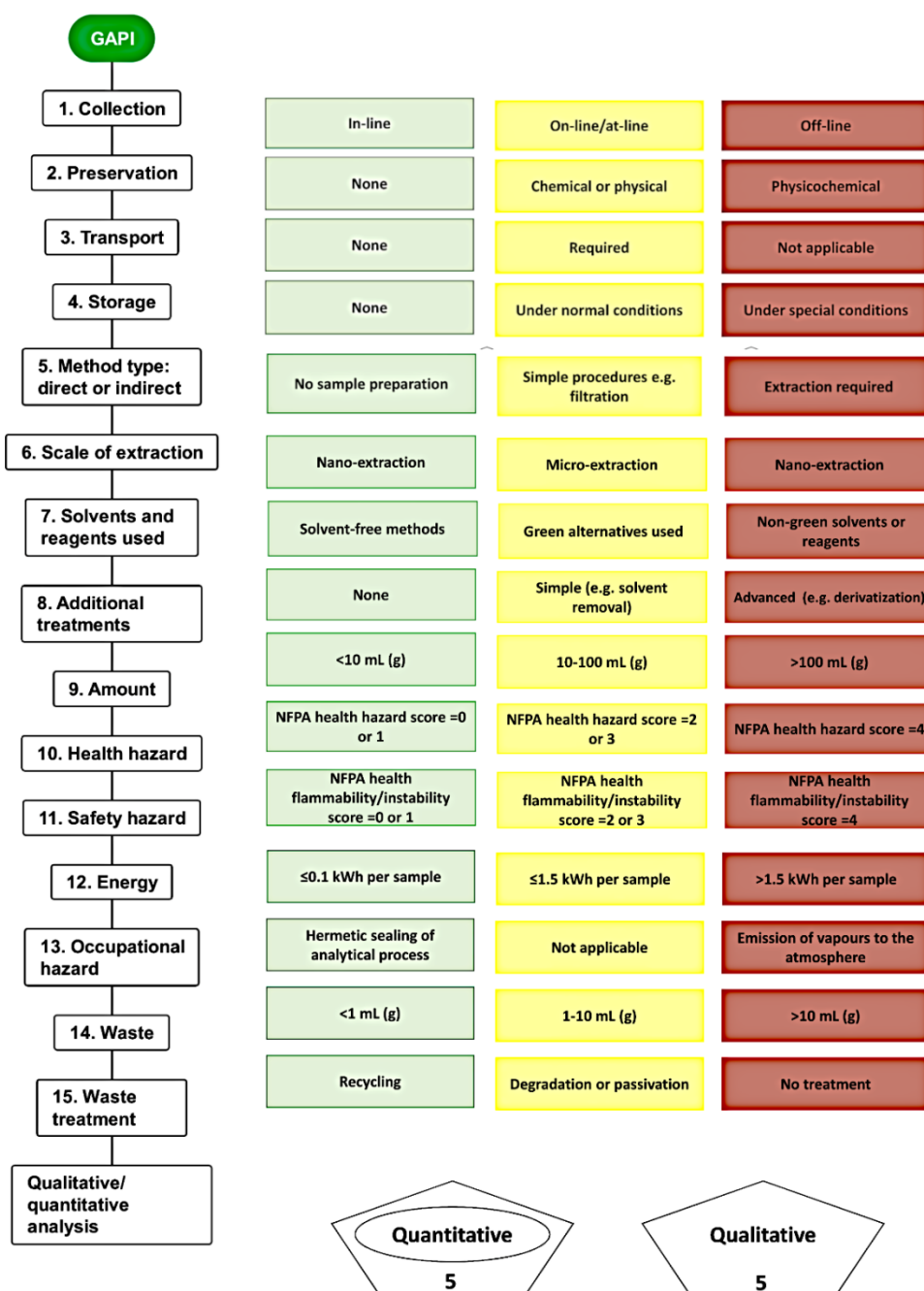


Figure 5. Color assignment in the GAPI approach. Different fields in the pictogram labeled with numbers 1-15 are filled with green, yellow, or red. The color of the boxes indicates in which case which color should be given to the corresponding field of the pictogram. The circle in the centre indicates the methods quantitative analysis (adapted from reference (15)).

Slika 5. Dodela boja u GAPI pristupu. Različita polja u piktogramu se obeležavaju brojevima od 1 do 15 i boje zeleno, žuto ili crveno. Boja polja određuje dalju boju koja se dodeljuje odgovarajućem delu piktograma. Krug u centru ukazuje da se metoda koristi za kvantitativnu analizu (prilagođeno iz reference (15)).

is omitted. In general, the more steps required, the less green the analytical method. Below is a brief explanation of Figure 5.

Another important part is the circle in the middle of the GAPI symbol. For methods intended for qualitative analysis only, the circle is omitted from the GAPI symbol, while it is included if the method is intended for quantitative analysis. GAPI thus provides information not only on the environmental friendliness of methods, but also on whether the method is suitable for the intended purpose of analysis. Comparison of methods with different purposes is not relevant.

The pictograms are convenient for users because they allow for quick visual comparison of different methods while providing thorough insight into different aspects of the method (15). More specifically, by visually examining the pictograms, we obtain not only a general assessment of the greenness of the method, but also an assessment of the environmental impact of each step, which allows us to identify critical aspects that can be considered in our future attempts to develop greener methods.

The Analytical Eco-Scale score

The Analytical Eco-Scale is an extensive approach based on penalizing parameters of the analytical method that differ from the ideal green analysis (5). The ideal green analysis is defined as one that uses no or minimal amounts of reagents, has minimal energy requirements, and generates no waste. Since this ideal case cannot usually be achieved with the exception of direct analytical methods, the concept of the analytical Eco-Scale score introduced somewhat looser criteria for the ideal green analysis, including the following: (i) the reagents used are not associated with physical, environmental, or health hazards; (ii) the energy consumption per sample is less than 0.1 kWh; and (iii) no waste is generated (5).

Penalty points are assigned for each considered parameter of the evaluated analytical method that deviates from the ideal green analysis (5). The point value for the ideal green analysis is 100. The analytical Eco-scale score of the evaluated method is calculated by subtracting the sum of the penalty points from 100. Based on the calculated score, methods can be classified as follows: (i) excellent green analysis (when the score is > 75); (ii) acceptable green analysis (when the score is >50); and (iii) inadequate green analysis (when the score is <50) (5).

The method parameters evaluated are the reagents, energy consumption per sample, occupational hazard, amount of waste generated, and waste management. Two main aspects are considered when evaluating the reagents: their physical, environmental, and health hazards, and their amount. The hazards associated with reagents can be evaluated based on the Environmental Protection Agency (EPA) documents, National Fire Protection Association (NFPA) classification, or GHS classification. An approach based on GHS pictograms and signal words is suggested in the reference (5). Reagents are usually labeled with one or more GHS pictograms that relate to different types of hazards. For each pictogram, one or two penalty points are assigned for the hazard of the reagent

depending on the signal word used. The GHS uses the signal words "danger" for a more severe hazard and "warning" for a less severe hazard. One penalty point is assigned for the less severe hazard, while two penalty points are assigned for the more severe hazard. The penalty points for the pictograms are added up to get the subtotal penalty points for reagent hazard.

On the other hand, the subtotal penalty points for the amount of reagents are assigned as follows: one penalty point for the amount <1mL (g), two penalty points for the amount 10-100 mL (g), three penalty points for the amount >100 mL (g). The total penalty points assigned to the reagents are calculated by multiplying the quantity-based penalty points by the hazard-based penalty points.

Penalty points are assigned based on energy consumption per sample as follows: no penalty points for consumption ≤ 0.1 kWh, one penalty point for consumption ≤ 1.5 kWh, and two penalty points for consumption > 1.5 kWh. Penalty points related to occupational hazard are not assigned if the analytical process is hermetically sealed, while three penalty points are assigned for analytical methods that cause emission of vapors and gasses into the air. Penalty points for waste are assigned as follows: (i) no waste - no penalty points; (ii) amount of waste less than 1 mL (g) - one penalty point; amount of waste 1-10 mL (g) - three penalty points; amount of waste greater than 10 mL (g) - five penalty points; waste recycling - no penalty points; waste degradation - one penalty point; waste passivation - two penalty points; no treatment - three penalty points. Further details and examples can be found in the reference (5).

The analytical Eco-Scale is a comprehensive, quick, and easy-to-use semi-quantitative tool with detailed criteria and guide for calculating score (5). The disadvantage of this tool is that it provides only general information, without any qualitative information (15). In other words, the result of the analytical Eco-Scale approach is a number and therefore does not imply any critical steps in the analytical procedure.

Analytical GREENness (AGREE)

AGREE is a novel greenness assessment tool that offers many advantages over the previously discussed tools. AGREE is a comprehensive tool because it includes 12 GAC principles as assessment criteria (16). Using appropriate functions, inputs related to these criteria are converted into scores, on a common scale from 0 for the least green to 1 for the greenest method with respect to the corresponding criterion. Moreover, AGREE is a flexible tool that allows assigning different weights to different inputs, as different purposes of analytical methods may impose the importance of compliance with different GAC principles (16). The weighted scores of each step are summed to obtain a total score for the analytical method. The total score can take values from 0 to 1. It is clear that a greener method will have higher total scores, but no threshold has been proposed, as in the case of the analytical eco-scale score. As shown in Figure 6, the output of AGREE is a simple clock-like symbol consisting of the total score placed inside a colored circle and a segmented rim surrounding the circle. Each segment, labeled with the numbers 1-12,

corresponds to a different GAC principle. The color of the segment indicates whether the method is green from the perspective of the corresponding GAC principle, while the color of the center indicates the overall greenness of the method considering all 12 GAC principles. The output described is simple, easy to read, understand and interpret. AGREE combines the positive sides of the analytical eco-scale score and the GAPI. Similar to the analytical eco-scale score, it is a quantitative tool that allows easy comparison of different analytical methods in terms of their total scores, and similar to GAPI, it includes a color-coded representation that clearly shows the causes of lower environmental friendliness of the methods, which can then be further addressed. It is also important to note that free, user-friendly software is available to enable the use of AGREE (16).

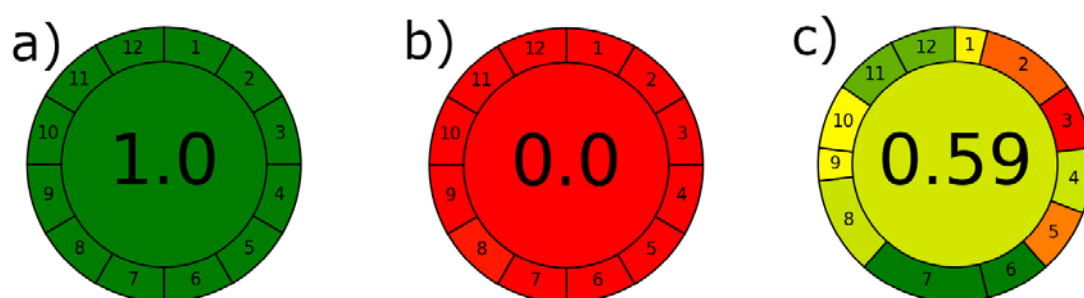


Figure 6. The result of the AGREE evaluation for: a) the ideal green method; b) the completely non-green method; and c) the moderately green method with adjusted weighting of the different criteria during the evaluation.

Slika 6. Rezultat AGREE procene za: a) idealnu zelenu metodu; b) potpuno ne-zelenu metodu; c) umereno zelenu metodu sa prilagodjenim težinama za različite kriterijume tokom procene metode.

Other approaches

HPLC-EAT (environmental assessment tool) is a simple and fast approach to evaluate the environmental friendliness of HPLC methods (12). Free software is available to calculate the HPLC-EAT score. However, only the hazards posed by the chemicals used in the analysis are considered when calculating the score (63). This approach is much less comprehensive than the approaches discussed previously.

More recently, a tool called the Complementary Green Analytical Procedure Index (ComplexGAPI) was introduced by Plotka-Wasyłka and Wojnowski (64). This is an extended GAPI approach that, in addition to assessing the greenness of all steps in the analytical procedure, also assesses the greenness of processes that are carried out prior to the analysis, such as the synthesis of nanoparticles, ionic liquids, or deep eutectic solvents. The symbol used in the ComplexGAPI evaluation consists of five pentagrams as in the GAPI, with an additional hexagon in the lower area reflecting the environmental friendliness of the processes performed before the analysis. This evaluation is simple and fast, as there is free software for ComplexGAPI (64).

When it comes to comparing a large number of available analytical methods for a given analyte in a given sample, e.g., 30, the previously described approaches become impractical. A suitable solution in these cases may be the use of multivariate analysis or multicriteria decision analysis (MCDA) (63). In multivariate analysis, variables related to environmental friendliness are used to group the analytical procedures. This can be done through cluster analysis, principal component analysis, and self-organizing maps. In the multivariate approach, the procedures are only grouped, while MCDA allows ranking of procedures (63). In MCDA, the objective of the analysis (greenness) and quantitative criteria related to the objective (greenness criteria) are first defined. The next step is to identify all alternative analytical methods for the purpose of interest and apply MCDA algorithms such as TOPSIS, PROMETHEE, or AHP to rank them (17). The result is given in the form of a score and is therefore easy to interpret (63).

White analytical chemistry (WAC)

The concept of sustainable development emphasizes the need to consider economic, social and environmental aspects in the development of projects and processes (65). The GAC approach is consistent with sustainable development, but it is incomplete because it does not take into account the social (analytical efficiency) and economic (economic/practical efficiency) dimensions of methods that should be considered when developing reliable "fit-for-purpose" methods. Therefore, the white analytical chemistry (WAC) approach was proposed as a holistic approach to implement sustainable development in analytical methods (18). WAC is based on the recently developed Red-Green-Blue (RGB) model, where red represents analytical efficiency, green represents environmental friendliness and safety, and blue represents economic/practical efficiency. Analytical efficiency refers to the degree to which the method meets the validation criteria and is very important because validation confirms that the results of the analysis are reliable and therefore can be used as a basis for decision making (66). The economic/practical efficiency depends on the total cost, simplicity, time required for the analysis, etc. Based on the RGB tool, the methods are evaluated and an overall color is assigned depending on the intensity of each primary color (red, green and blue). A high and uniform saturation of the individual colors results in an overall white color. Therefore, "whiteness" can be used as a quantitative parameter reflecting how balanced the three main pillars of sustainable methods are.

Nowak et al. proposed 12 principles of WAC along with the more detailed RGB model called RGB 12 (18). The WAC principles are divided into three categories - red, green and blue, with each category containing four principles. The four green principles are actually the sum of 12 GAC principles and are therefore no less comprehensive. In addition, some GAC principles are related to the blue principles, such as integration, automation, reduced sample size requirements, etc. The 12 principles from WAC along with the output of the RGB 12 tool are shown in Figure 7. The RGB 12 model is easy to use and freely available in the form of an Excel spreadsheet. It allows values between 0 and 100 to be entered in the fields corresponding to each WAC principle. A value of 100

should be assigned if the method fully meets the requirements for the intended purpose with respect to the observed WAC principle (18).

Disadvantages of the RGB-12 tools include the potential subjectivity of the authors, lack of a consistent approach to assigning scores, and lack of a weighting option based on the importance of the parameter (18). However, as an alternative to weighting, scores can be assigned so that small changes in the parameter being evaluated result in large changes in scores.



Figure 7. a) The 12 principles of white analytical chemistry (WAC); The result of the RGB 12 assessment for: b) a method that agrees with all WAC principles; c) a method that does not agree with any of the WAC principles.

Slika 7. a) 12 principa bele analitičke hemije (WAC); Rezultat RGB 12 procene za: b) metode koja je u saglasnosti sa svim WAC principima; c) metode koja nije u saglasnosti ni sa jednim WAC principom.

Conclusion

This article provides a brief overview of GAC principles, the main drawbacks of HPLC with respect to GAC principles, approaches used to achieve greener HPLC analysis, and various tools for assessing greenness. The greening of HPLC methods is of great importance in view of the quantities of waste produced and their negative impact on the environment. HPLC analysis can be improved in terms of greenness through one

or more of the approaches described. However, after each modification, greenness should be evaluated to confirm that the modified method is indeed greener than the original method.

White analytical chemistry is an interesting concept in line with sustainable development, which includes not only the environmental friendliness of the analytical method, but also its economic/practical aspect and the performance of the analytical method for its intended purpose, i.e., social requirements. Since the purpose of chemical analysis is to provide information needed for decision making, we should be cautious about sacrificing the performance of the method for its greenness. An analytical method in the pharmaceutical industry must meet defined validation criteria for its intended purpose, as the results of the analysis form a basis for marketing authorization, batch release, etc. Therefore, we propose the use of the RGB 12 approach or greenness assessment of previously validated methods. Among the approaches for assessing environmental friendliness, AGREE seems to be the most promising, as it is the most comprehensive tool that is also flexible, easy to use, and provides clear but very informative output to readers.

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Upravljanje resursima u HPLC: Otkrivanje zelenog lica farmaceutske analize

Jevrem Stojanović*, Jovana Krmar, Biljana Otašević, Ana Protić

Univerzitet u Beogradu – Farmaceutski fakultet, Katedra za analitiku lekova,
Vojvode Stepe 450, 11010 Beograd, Srbija

*Autor za korespondenciju: Jevrem Stojanović, e-mail: jstojanovic@pharmacy.bg.ac.rs

Kratak sadržaj

Tečna hromatografija pod visokim pritiskom (HPLC) je tehnika od ogromne važnosti u analitici lekova zbog svoje mogućnosti da razdvoji umereno do manje polarna jedinjenja, kao što su aktivne farmaceutske i srodne supstance. Koncept zelene analitičke hemije (GAC) ima za cilj da obezbedi ekološki prihvatljivije i bezbednije metode u pogledu reagenasa, energije i otpada. Jedan od glavnih izazova GAC je da pronađe odgovarajući pristup za procenu „zelenosti“ analitičkih metoda. Proširenje GAC, nazvano bela analitička hemija (WAC), uvedeno je kako bi se uzela u obzir ne samo ekološka prihvatljivost, već i drugi aspekti koji doprinose održivosti metoda, kao što su analitička i ekonomska ili praktična efikasnost. HPLC metode suštinski nisu „zelene“ zbog obimne potrošnje toksičnih organskih rastvarača i posledičnog stvaranja velikih količina toksičnog otpada. Srećom, postoji mnogo pristupa za prevazilaženje ne-zelene prirode HPLC metoda. U ovom radu su predstavljene različite modifikacije HPLC metode koje povećavaju ekološku prihvatljivost, kao i različiti alati koji se koriste za procenu ekološke prihvatljivosti. Pored toga, predstavljen je novi koncept bele analitičke hemije.

Ključne reči: tečna hromatografija visokih performansi, procena ekološke prihvatljivosti metoda, održivost
